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13. ABSTRACT *(Maximum 200 words)*

The long term goal of this project is to characterize molecular and cellular mechanisms responsible for aromatase expression surrounding a breast tumor. We have previously detected the highest levels of adipose tissue aromatase transcript levels proximal to a tumor compared with distal sites in the breast. During the past year, the following studies have been completed: We have demonstrated that the highest aromatase transcript levels and fibroblast-to-adipocyte ratios are found in lateral breast regions in a group of disease-free women undergoing reduction mammoplasty. This distribution pattern directly correlates with the most common site of carcinoma in the breast. We have also developed a novel competitive RT-PCR/PAGE method using multiple internal standards in order to quantify aromatase transcript populations with unique untranslated 5'-ends in a tissue. Using this technology, aromatase promoters preferentially used in adipose tissue proximal to a tumor will be determined in mastectomy samples. In a separate study, we have demonstrated that tumor-conditioned culture media were able to markedly stimulate aromatase expression in adipose fibroblasts. Next, we identified at least a group of these tumor-derived aromatase-stimulating factors as members of the IL-6 cytokine family. Finally, we described a role for a Jak/STAT signalling pathway in adipose fibroblasts, which mediates stimulation of aromatase by these cytokines.

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Serdar E. Bulun 10/7/95

PI - Signature Date

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5. INTRODUCTION

I prepared this Annual Report as a requirement of the Career Development Award entitled "Breast Cancer and Estrogen Biosynthesis in Adipose Tissue" funded by the US Army Medical Research and Material Command Breast Cancer Research Program. This report covers research for the period 9/12/94-9/12/95. The long term goal of this Application was to characterize the molecular and cellular mechanisms responsible for estrogen synthesis in adipose tissue surrounding a breast tumor. Since aromatase P450 (P450arom) catalyzes the conversion of C_{19} steroids to estrogens, our studies focus on the expression of P450arom in adipose fibroblasts in relation to a tumor. During preliminary studies, we have detected the highest levels of adipose tissue P450arom transcripts in breast quadrants bearing a tumor compared with tumor-free quadrants.

The grant proposal included four specific aims: The **first aim** is to quantify adipose tissue P450arom transcript levels in an additional number of mastectomy specimens at various distances from the tumor using a novel quantitative RT-PCR method. We also proposed to quantify these transcripts in breast samples of women undergoing reduction mammoplasty to ascertain the distribution of aromatase expression. This aim also included determining the ratio of fibroblasts, the aromatase-expressing cell type, to mature adipocytes in these tissue samples. A substatial part of this specific aim has been accomplished as indicated below in **6. BODY**. The **second specific aim** included determination of the promoter regions used to express aromatase in adipose tissue samples proximal to a tumor. This involves a novel quantitative RT-PCR method to simultaneously determine untranslated 5'-ends of P450arom transcripts in adipose tissue samples using 4 different internal standard comlementary (c) RNAs. Since this involved preparing homologous internal standard cRNAs and constructing multiple standard curves, developing this novel method itself constituted a time-consuming study and lead to a peer-reviewed publication. We are currently applying this method to determine the distribution of 5'-ends of P450arom transcripts in adipose tissue samples from mastectomy specimens. **Specific Aim 3** involves characterization of novel 5'-ends of P450arom transcripts in breast cancer tissues and aromatase-expressing cancer cell lines, *e.g.*, MCF-7. This involves using rapid amplification of cDNA ends, and sequecing to identify potential P450arom promoter regions which have not yet been described. For this purpose, we currently collect tissues and identify suitable aromatase-expressing cell lines. Finally, the **fourth specific aim** was to determine whether secretory factors of breast cancer cells induce aromatase expression in the surrounding adipose tissue and to characterize such factors. These goals have been accomplished and two related studies have been published in peer-reviewed journals, whereas part of the data related to expression of specific secretory products by breast tumors await publication.

6. BODY

I. The following study has been completed in reference to **Specific Aim 1**. An abstract has been published (1), and the manuscript is in press (2).

In disease-free breasts, the highest fibroblast-to-adipocyte ratios and P450arom transcript levels were found in the outer regions (1,2)**.** We determined distribution of P450arom mRNA levels and fibroblast to adipocyte ratios in disease-free breasts of premenopausal women between the ages of 19 and 42. Adipose tissue samples were collected from 3 regions of both breasts in 13 women undergoing reduction mammoplasty. Samples were taken from midportions of outer and inner regions, as well as from the midline above the nipple (designated as upper). Mode of sampling was dictated by the technique of surgery. Total RNA was isolated (n=11), and an hematoxylin and eosin-stained section was prepared (n=12) from the same sample from each region of both breasts. Overall, 67% of the highest fibroblast to adipocyte ratios and 64% of the highest P450arom transcript levels were detected in an outer breast region, whereas in only one patient, the highest values were detected in an inner region. Parametric analysis of variance (ANOVA) showed significant differences between the fibroblast content of the regions [p(F)=0.037]. This distribution pattern directly correlates with the most common or the least common sites of carcinoma in the breast in large series, which are the outer and inner regions, respectively. (In 11 of 15 patients of our previous study (3) and 10 of 12 patients in O'Neill's study (4), the tumors were found in a lateral quadrant.) Moreover, a direct relationship was demonstrated between adipose fibroblasts and P450arom transcripts within the breast, in that regions with the highest fibroblast to adipocyte ratios contained the highest P450arom transcript levels (ANOVA of contrast variables, p=0.0009). This suggests that, similar to our previous findings in the breast bearing a tumor, adipose tissue aromatase expression in the disease-free breast is also determined by the local ratio of fibroblasts to adipocytes. We further conclude that since breast cancer occurs in regions of the breast with the highest levels of aromatase expression, the presence of high fibroblast content and P450arom transcript levels in the outer region of the disease-free breast may be of pathophysiologic significance in the development of breast cancer.

II. To accomplish the studies proposed in **Specific Aim2**, we first developed a novel competitive RT-PCR/SDS-PAGE method to quantify promoter-specific P450arom transcripts with unique 5'-ends. The manuscript describing the following study is in press (5):

Quanfication of alternatively spliced transcripts of the P450arom gene in aromatase-expressing human cells (5). Here, we describe a competitive RT-PCR/PAGE method to quantify three major 5'-termini of P450arom transcripts, *i.e.* promoter II-specific (PII), exon I.3- and exon I.4-specific. Since placental promoter specific exon I.1 is not present in other tissues, we did not attempt to amplify exon I.1-containing transcripts in adipose tissue samples. Using this method, we were able to

quantitatively detect multiple transcripts present in primary cultures of human adipose fibroblasts, adipose tissues and ovarian granulosa cells grown in different culture conditions. Most importantly, this method is applicable to quantitative analysis of alternatively spliced transcripts of other genes in which this form of regulation of expression is utilized. This method is summarized in Fig. 1.

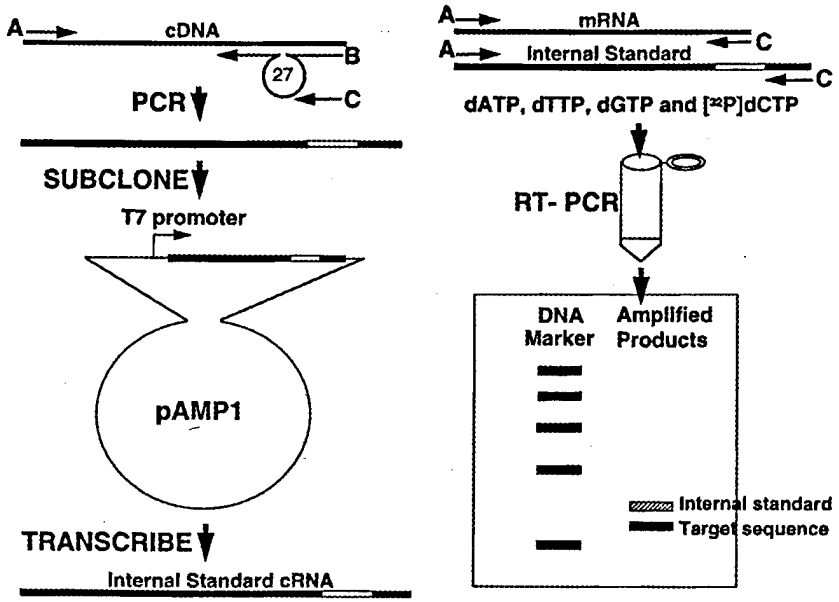

Figure 1. ***Synthesis of a reference internal standard cRNA for quantifying human target transcripts by the looped oligo method (left panel) and RT-PCR of this cRNA together with total RNA for quantification of target transcripts (right panel). The bold lines indicate the coding region of a target transcript or cDNA desired to be quantified in the tissue total RNA sample. A, B and C are oligonucleotides for PCR. A and B are used in generating an internal standard sequence. The loop in oligonucleotide B represents an extra non-target sequence of 27 nucleotides, which is represented by the blank line in the internal standard sequence. A very small quantity (0.1-1pg) of the internal standard cRNA is coamplified and labeled by RT-PCR together with the target transcript in total RNA from a tissue sample (right panel). Amplified fragments are fractionated by PAGE, and radioactivity on bands is quantified. Ratio of the value for the shorter band (target transcript) to the longer band (internal standard) is recorded as an arbitrary unit for the tissue level of that target transcript.***

First, we have generated 4 internal standard cRNAs specific for 3 untranslated 5'-ends and a coding region using the "looped oligo" technique (Fig. 1). The generated cRNAs differed from the wild type mRNA sequence by an extra 27 bp-long AT-rich region. A known copy number of these internal standards was added to each RT-PCR reaction containing target RNA from various tissues, *e.g.*, the adipose tissue. [^{32}P]-labelled dCTP was used in amplification reaction and the products were analyzed on 4% non-denaturing polyacrylamide gels. Radioactivity of bands were quantified by a PhosphorImager. Ratio of radioactivity of the smaller-size band (target mRNA) to that of the larger-size band (internal standard cRNA) was expressed as an arbitrary unit for the level of a P450arom transcript population with a promoter-specific 5'-end. Total P450arom mRNA level in each

sample was also quantified using an internal standard cRNA corresponding to the coding region only. In a tissue sample, we sought to demonstrate that the levels of PII-, I.3- and I.4-specific transcripts approximately added up to the total transcript levels determined by amplifying the coding region. Therefore, we have performed a number of experiments to optimize internal standard quantities and PCR conditions. These are detailed in the manuscript which is currently in press (5).

III. Studies proposed in **Specific Aim 3.** We are currently collecting tumor specimens and identifying breast cancer cell lines which express adequate levels of aromatase in order to determine possible novel 5'-ends of P450arom transcript in these cells using RACE.

IV. The following studies were completed and a major portion of these studies have been published. As indicated in **5. INTRODUCTION**, the goals included in the broad category **Specific Aim 4** have been accomplished. However, we are following our scientific instincts to continue our studies in the direction of further characterizing the regulation cytokine expression in the breast cancer. We will continue to explore the roles of IL-6 cytokine family in local estrogen production and peri- and intra-tumoral fibroblast proliferation, *i.e.*, the desmoplastic reaction.

***Serum-free conditioned medium from the human breast cancer cell line, T47D dramatically stimulates aromatase activity of glucocorticoid-treated adipose fibroblasts in monolayer culture* [*Fig. 2* (6)].** In our laboratory, dexamethasone (DEX) was previously found to increase aromatase activity in adipose fibroblasts in culture in a concentration- and time-dependent fashion (7). The presence of serum in the culture medium was mandatory for this effect of glucocorticoids to be manifest, since under serum-free conditions, the stimulatory effect of DEX was greatly reduced. We designed an experiment to determine whether serum can be replaced by secretory products of breast cancer cells in stimulating aromatase activity of DEX-treated adipose fibroblasts: the T47D breast cancer cell line was used for conditioning of serum-free, phenol red-free Waymouth's media. Separately, adipose fibroblasts in culture were maintained in serum-free Waymouth's media for 24 h. Thereafter, adipose fibroblasts were incubated with conditioned serum-free media from T47D cells and DEX ($2.5X10^{-7}M$). Aromatase activity was measured after 24 h. Serum-free conditioned medium from breast cancer cells mimicked fetal bovine serum (10%) to induce aromatase activity of DEX-treated adipose fibroblasts (Fig. 2). Treatment of T47D cell with E_2 potentiated this effect in a dose-dependent fashion. Heat inactivation destroyed the stimulating ability of conditioned medium. The majority of P450arom 5·-termini expressed by adipose fibroblasts incubated with conditioned medium plus dexamethasone contained promoter I.4-specific sequence. We also observed that adding anti-IL-11 antibodies to T47D-conditioned medium abolished its stimulatory effect on aromatase expression in adipose fibroblasts (our unpublished observations). Additionally, we found that conditioned medium from adipose fibroblasts themselves was also efficacious in substituting for serum to stimulate aromatase expression.

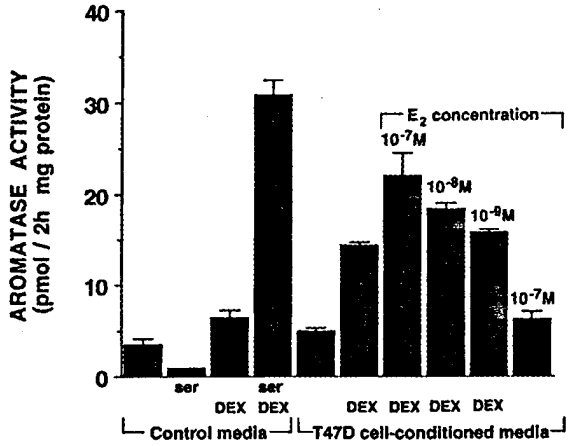

Figure 2. ***Effects of DEX ($2.5X10^{-7}M$) plus serum-free conditioned media from T47D breast cancer cell line on aromatase activity of adipose fibroblasts in culture. E_2 (10^{-7}-$10^{-9}M$) treatment of cancer cells increased the stimulatory effect of the conditioned media in a dose-dependent manner. Note that E_2, by itself does not change aromatase activity of the adipose fibroblasts.***

Aromatase activity of glucocorticoid-treated adipose fibroblasts in a monolayer co-culture model was markedly stimulated after insertion of T47D cells into this system (our unpublished observations). Adipose fibroblasts and T47D breast cancer cells were co-cultured using cell culture inserts containing membranes with 0.45 μm pores. T47D cells introduced in culture inserts into the serum-free, phenol red-free co-culture system stimulated aromatase activity of glucocorticoid-treated adipose fibroblasts. In this regard, presence of T47D cells mimicked the stimulatory effect of serum on glucocorticoid-treated adipose fibroblasts. E_2 treatment of cancer cells potentiated this stimulatory effect.

***Role of the IL-6, IL-11, LIF, OSM cytokine family and a Jak/STAT pathway in regulation of the adipose-specific P450arom promoter, I.4* [*Fig. 3* (8)].** We recently discovered that a Jak/STAT signalling pathway mediates the stimulation of expression of the P450arom gene in human adipose tissue. P450arom expression in adipose fibroblasts maintained in the presence of serum and glucocorticoids is regulated by promoter I.4 which lies at least 20 kb upstream of the translation start site. I.4 is a TATA-less promoter, which contains a GRE, an Sp1 binding site and a GAS (interferon-γ Activation Site) element. Furthermore, the stimulatory action of serum or of conditioned media can be mimicked by IL-11, LIF and OSM (Fig. 3), as well as by IL-6, provided the IL-6 soluble receptor is also present. Stimulation of the adipose fibroblasts by these factors led to rapid phosphorylation of Jak1, but not Jak2 or Jak3, on tyrosine residues. STAT3, but not STAT1 or ISGF3, was also phosphorylated and bound to the GAS element in the I.4 promoter region. When regions of this promoter were fused upstream of the CAT reporter gene and transfected into adipose fibroblasts, mutagenesis or deletion of the GAS element led to complete loss of reporter gene expression. We conclude that stimulation of aromatase expression by members of the IL-6 cytokine subfamily through this Jak/STAT pathway appears to be a major mechanism regulating estrogen biosynthesis in elderly women.

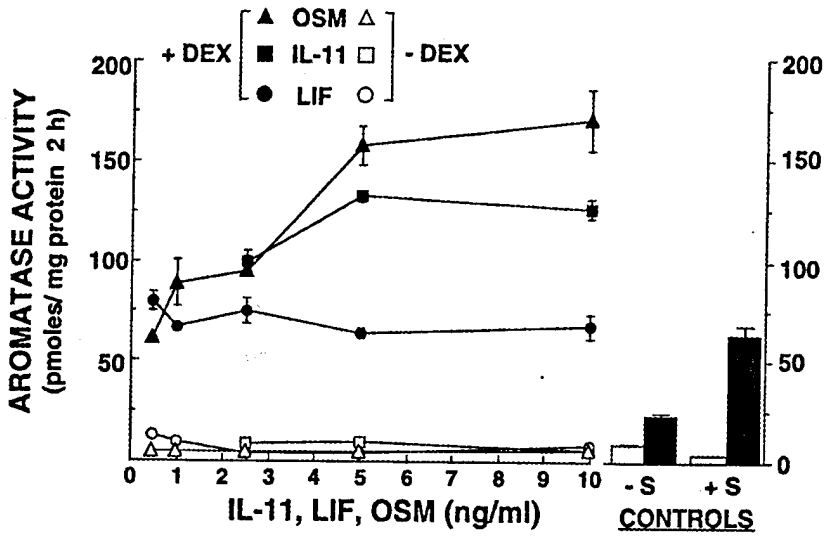

Figure 3. ***Concentration-dependence of the actions of LIF, IL-11 and OSM to stimulate aromatase activity of adipose fibroblasts in the absence (open symbols) or presence (solid symbols) of dexamethasone (DEX, 2.5x10^{-7} M). Confluent adipose fibroblasts in primary culture were maintained for 24 h in the presence or absence of DEX, LIF, IL-11, or OSM in various concentrations. Control dishes incubated in the presence or absence of serum are indicated as ± S, with open bars indicating the absence and solid bars the presence of dexamethasone.***

Expression of IL-6 and IL-11 in adipose fibroblasts, T47D breast cancer cells and breast cancer tissues (our unpublished observations). Using RT-PCR, we detected IL-6 and IL-11 transcripts in T47D breast cancer cells in culture. Moreover, the transcript levels of IL-11 increased by several fold after E_2 (10^{-8} M) treatment of T47D cells. IL-6 and IL-11 transcripts were detected in four breast cancer tissue samples. Transcripts of these cytokines were also present in adipose fibroblasts in culture. Thus far, LIF or OSM transcripts have not been detected in these tissues or cells.

A case study of aromatase overexpression in adipose tissue (9). We studied the mechanism of estrogen production in a 17-year-old boy with a history of prepubertal gynecomastia since 7 years of age and circulating estrogen levels within the range of an ovulating woman. Tumors of the adrenals and testes were excluded by MRI scan and testicular biopsies. Results of the laboratory tests were as follow: Karyotype 46,XY; peripheral E_2 130-250 pg/ml; E_1 390-570 pg/ml; testosterone 0.97-1.33 ng/ml; androstenedione 0.49 ng/ml; FSH <2 mIU/ml; LH 3-5.8; βHCG <5. The transfer constant of conversion of plasma androstenedione to estrone was 40-50 times that of normal subjects. Testicular and adrenal vein sampling indicated that all of E_1 and E_2 in this boy were formed in extraglandular sites from plasma androstenedione and testosterone. A defect of the P450arom gene was ruled out by direct sequencing. Next, we quantified P450arom transcripts in the buttock and thigh adipose tissue biopsy samples. Transcript levels were found to be 10 times higher than those of a normal 16-year-old boy. Most of the transcripts appeared to contain I.4 and I.3-specific sequences. Our results suggest

extensive extraglandular aromatization in this boy results from high levels of aromatase expression in the adipose tissue. Since the sequence of the gene appears to be normal, then the high level of expression may be the consequence of a defect in the signalling pathway regulating aromatase expression in adipose tissue. This case exemplifies the extremely high estrogen producing potential of human adipose tissue.

Determination of promoter usage for aromatase expression in an feminizing adrenal tumor from an adult man (10). Promoter II was found to be primarily used for extremely high levels of aromatase expression in a rare adrenal tumor from an adult man (10).

7. CONCLUSIONS

Based on the above considerations, we propose the following hypothesis: regional differences in relative proportions of histologic components of the breast adipose tissue (*e.g.*, fibroblasts vs. mature adipocytes) may be the primary cause of estrogenic concentration gradients, since regions containing higher numbers of fibroblasts are the sites of increased aromatase expression. Although the initiating events are unknown, malignant cells in the regions displaying higher P450arom expression are more likely to proliferate. Secretory products of the tumor stimulated by estrogens may in turn further increase aromatase expression in the surrounding adipose tissue. These products will additionally stimulate proliferation of aromatase-expressing fibroblasts to generate a fibrous capsule around the tumor, *i.e.,* desmoplastic reaction. Estrogens will continue to positively influence neoplastic growth by increasing the expression of secretory products and their receptors in the tumor tissue. Thus a positive feed-back loop is established in which locally-produced estrogens and tumor-derived factors, *e.g.*, members of IL-6 cytokine family, act by paracrine and autocrine mechanisms to sustain the growth and development of the tumor.

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Publications by the Principal Investigator which are relevant to this research effort and which acknowledge this grant are listed:

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9. APPENDIX

Copies of the above publications, indicated with *, are included in this annual report.

DISTRIBUTION OF AROMATASE P450 TRANSCRIPTS AND ADIPOSE FIBROBLASTS IN THE HUMAN BREAST

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ABSTRACT

The fibroblast component of adipose tissue is the primary extraglandular site of aromatase P450 (P450arom) expression, which is responsible for the conversion of C_{19} steroids to estrogens. Previously, we have shown positive correlations between the level of P450arom transcripts and the ratio of fibroblasts to mature adipocytes in adipose tissue samples proximal to breast tumors. The present study was conducted to determine distribution of P450arom mRNA levels and fibroblast to adipocyte ratios in disease-free breasts of premenopausal women. Adipose tissue samples were collected from 3 regions (outer, upper and inner) of both breasts of 13 women undergoing reduction mammoplasty. Histologic composition of adipose tissue (n=12) was determined by morphometry using a computerized image analysis program. We used a competitive reverse transcription-polymerase chain reaction method employing a rat P450arom cRNA as an internal standard to quantify adipose P450arom transcripts in tissue total RNA samples (n=11). Overall, 67% of the highest fibroblast to adipocyte ratios and 64% of the highest P450arom transcript levels were detected in an outer breast region, whereas in only one patient, the highest values were detected in an inner region. Parametric analysis of variance (ANOVA) showed significant differences between the fibroblast content of the regions [p(F)=0.037]. This distribution pattern directly correlates with the most common or the least common sites of carcinoma in the breast, which are the outer and inner regions, respectively. Moreover, a direct relationship was demonstrated between adipose fibroblasts and P450arom transcripts within the breast, in that regions with the highest fibroblast to adipocyte ratios contained the highest P450arom transcript levels (ANOVA of contrast variables, p=0.0009). These results suggest that, similar to our previous findings in the breast bearing a tumor, adipose tissue aromatase expression in the disease-free breast is also determined by the local ratio of fibroblasts to adipocytes.

INTRODUCTION

Conversion of C_{19} steroids to estrogens by aromatase P450 (P450arom) primarily takes place in a number of human cells, *e.g.*, the ovarian granulosa cell (1), the placental syncytiotrophoblast (2), and the adipose fibroblast (3,4). Aromatase expression does not occur in the mature adipocytes but rather in the fibroblasts which are dispersed between the adipocytes (3,4). Adipose fibroblasts are believed to be the preadipocytes which give rise to mature adipocytes in human adipose tissue. Aromatase expression in adipose tissue has long been implicated in the pathophysiology of breast cancer growth (5-8). Potent aromatase inhibitors have recently been widely used in the treatment of postmenopausal breast cancer (9-11). Peripheral estrogen production in whole body adipose tissue may be enough to effectively increase circulating levels of estrogens (5). Estrogen produced by adipose tissue within the breast also may act locally to promote growth of breast tumors: O'Neill and coworkers (7) demonstrated that the breast quadrant displaying the highest level of aromatase activity was consistently involved with tumor. Furthermore, we found the highest levels of P450arom transcripts in fat from the quadrants bearing tumors in 69% of the cases (8). In the same study, the quadrants containing the highest fibroblast to adipocyte ratios had the highest P450arom levels.

The present study was conducted to ascertain the distributions of adipose fibroblast content and P450arom transcript levels in tumor-free breasts of women undergoing reduction mammoplasty. We have previously demonstrated that both aromatase activity (3) and P450arom transcript levels (4) primarily reside within the fibroblast fraction of the adipose tissue. And we have observed a positive correlation between fibroblast to adipocyte proportions and P450arom transcript levels in breast adipose tissue from mastectomy specimens bearing tumors (8). However, the presence of a tumor in breast adipose tissue in this instance may likely have influenced the distribution of P450arom expression in the breast. Therefore, the present study used reduction mammoplasty samples to investigate the distribution patterns of fibroblasts and P450arom transcript levels in the disease-free breast of premenopausal women. Additionally, we investigated the possible effects of side (left *vs.* right) or position (outer, upper, inner) on these distribution patterns.

MATERIALS AND METHODS

Tissue acquisition and processing. Breast fat was obtained at the time of surgery from both breasts of 13 women undergoing reduction mammoplasty for symptomatic macromastia (6 samples per patient). Written consent was obtained prior to surgery including a consent form and protocol approved by the Institutional Review Board for Human Research of The University of Texas Southwestern Medical Center. Whole breast adipose tissue samples were frozen in liquid nitrogen and stored at -70°C.

Adipose tissue samples were obtained from the mid portions of the outer and inner breast halves. A third sample was also obtained from the middle of the upper breast fat, which practically is the mid point between the outer and inner samples above the nipple. This mode of sampling was dictated by the technique of the reduction mammoplasty operation.

Human RNA isolation and rat P450arom cRNA synthesis. Human total RNA was isolated from frozen tissue of 11 women by the guanidinium thiocyanate-cesium chloride method (12). Rat P450arom complementary (c) RNA was synthesized as previously described (4,8).

Quantitative PCR following reverse transcription (RT-PCR) and hybridization. RT-PCR using synthesized oligonucleotides as primers and radiolabelled probes was performed as previously described (4,8). Briefly, an antisense oligonucleotide complementary to coding exon IV was used for primer extension. The created single stranded P450arom cDNA template was then amplified by PCR using the same antisense oligonucleotide and a sense oligonucleotide identical to a sequence in coding exon II. As an internal standard, 1 pg of a homologous rat P450arom cRNA (4,8) was reverse transcribed and coamplified in each human breast total RNA sample in order to control and correct for the differences in amplification efficiency between these samples. The antisense and sense primers were designed as such to recognize identical sequences in both human and rat cDNAs. Amplified products were divided into half and transferred to separate membranes. Each membrane was hybridized with a species-specific, labeled oligonucleotide probe designed to recognize the heterologous midportion of the amplified human or rat product. An AMBIS image acquisition and analysis system was used to quantify radioactivity on each membrane. Since each sample initially

contained an equal amount of the rat cRNA, the ratio of human to rat amplification product obtained for the P450arom mRNA level in each adipose tissue sample was reported as an arbitrary unit.

Histology. Representative sections were obtained from each breast region (n=12) using the same frozen adipose tissue fragments, parts of which were previously used for RNA isolation. Sections (3μ-thick) of paraffin embedded tissue were stained with hematoxylin eosin. Adipocytes, fibroblasts, acellular stroma, ductal epithelium and endothelium-lined space (ELS) components were quantified by a computer-assisted image processing program (NIH, Image 1.51, Bethesda, MD) and were expressed as percentages of total image in any particular field.

Statistical Analysis. Parametric repeated measures analysis of variance (ANOVA) followed by Newman-Keuls multiple comparisons test were used to compare fibroblast proportions and P450arom transcript levels between regions of individual breasts. Multivariate ANOVA for repeated measures and ANOVA of contrast variables were used to test the correlation between fibroblast proportions and P450arom levels. Simple linear regression analysis was used in establishing the standard curve.

RESULTS

Standard curve (Fig. 1). Initially, we established a range of starting amounts of human breast adipose total RNA (2-16 μg) which yielded linear increases in amplification product, in the presence of a constant amount of coamplified rat P450arom cRNA (Fig. 1). The arbitrary units for P450arom mRNA levels were plotted against the initial breast adipose tissue total RNA quantities to create a standard simple regression line ($r=0.987$, $p<0.0001$). The samples were amplified in duplicate and mean value for each duplicate was reported as the P450arom transcript level for each sample. Individual values of the standard curve experiment remained within ± 15-25% of the mean. This experiment was repeated twice with similar results. Using individual values instead of means of duplicates did not change the precision of this assay. Subsequent experiments employed 10 μg of breast adipose RNA and 20 cycles of amplification, since under these conditions the intensity of the radioactivity on each slot fell within the linear range of increase in the human amplification product.

P450arom transcript levels in breast regions (Fig. 2, Table 1). Fig. 2 illustrates a representative

experiment for one of the 11 patients. Eleven such separate experiments were performed, and P450arom transcript levels are listed in Table 1. P450arom transcript levels from 6 breast regions (3 on each side) were compared for each woman. P450arom transcripts were detected in all samples by autoradiography and by the AMBIS Radioanalytic Imaging System. Parametric repeated measures ANOVA revealed a linear trend with the highest levels found in the outer region followed by the upper and the lowest levels in the inner region, however this relationship did not reach statistical significance [p(F)=0.110]. In 64% of women, the highest levels were localized to an outer breast region, whereas in only one patient, the highest level was found in an inner region. This distribution followed the same order as the fibroblast to adipocyte ratio distribution among the breast regions (See below).

Distribution of fibroblasts in breast regions (Fig. 3, Table 2). Sections of breast regions from 12 women revealed marked differences in the ratio of fibroblasts to adipocytes (1%-15%) (Fig. 3). Mature adipocytes comprised the rest of the sections. Glandular epithelium, vascular endothelial cells, and acellular stroma were noted in some samples in negligible proportions. Fibroblast to adipocyte ratios are listed in Table 2. Sixty seven percent of the highest ratios were found in an outer breast region, whereas only one patient had the highest fibroblast to adipocyte ratio in an inner region. Parametric repeated measures ANOVA revealed significant differences between regions of individual breasts [p(F)=0.037]. Newman-Keuls multiple comparisons test showed significant differences between the outer and inner or outer and upper regions ($p<0.5$), but no significant difference was found between upper and inner regions.

P450arom transcript levels and fibroblast content were determined in the identical breast adipose tissue samples of 10 women. There was a statistically significant direct relationship between the distributions of fibroblasts and P450arom mRNA levels in the breast. In other words, proportions of fibroblasts in the 6 breast regions of a woman increased in the same order as the P450arom transcript levels. Multivariate ANOVA comparing regional fibroblast to adipocyte ratios with regional P450arom mRNA levels revealed a direct relationship ($p=0.023$). Additionally, ANOVA of contrast variables showed that regional fibroblast to adipocyte ratios in the breast increased with significant differences between each other when they were arranged to match to regions with increasing order of P450arom

mRNA levels ($p=0.0009$). No influence of side (left vs. right) was noted on distribution of P450arom transcripts or fibroblasts.

DISCUSSION

Adipose tissue has a similar uniform histologic appearance in various parts of the human body; however, evidence from several laboratories including ours suggests endocrinologic behavior of this tissue may be different in the breast, abdomen, buttock, and thigh of a woman (13-15). Differences in adipose tissue aromatase expression at various body sites of women has been well characterized. Aromatase expression in adipose tissue samples of women was found to be the highest in the buttock region followed by the thigh and the lowest in the abdomen (13). Thus far, breast adipose tissue aromatase expression has not been directly compared to other body sites. We have found that different promoters are responsible for aromatase expression in adipose tissue in breast and lower thighs (1). This suggests different signal transduction pathways are responsible for aromatase expression at these two sites. Finally, in contrast to variable expression in breast regions, marked differences found in aromatase expression in the abdomen, buttock and thigh is not related to the fibroblast to adipocyte ratios at these body sites (our unpublished observations). This suggests that, in contrast to breast regions, intrinsic differences in the specific aromatase expression between the fibroblasts from these other body sites rather than fibroblast numbers determine local estrogen biosynthesis.

The studied group of women undergoing reduction mammoplasty suffered symptomatic macromastia. The etiology of this condition is not known, and it is possible that breast aromatase expression in these women may be significantly different than in women with smaller breasts. Therefore, our results should be viewed with a degree of caution when applied to the general population. Nevertheless, the subjects in this study represent a premenopausal group of women with cancer-free breasts, in contrast to our previous study group which was comprised of postmenopausal women with breast cancer (8). Theoretically, general anesthesia and its associated stress may give rise to elevated cortisol levels which may effect aromatase expression in adipose tissue. However, pharmacologic doses of glucocorticoids are capable of causing a detectable increase in aromatase activity of adipose fibroblasts after 3 hours of treatment (16), whereas the samples for this study were collected within one hour after the initiation of general anesthesia. Therefore, an effect of general aneshesia on our results through this mechanism is very unlikely. Finally, in this study, P450arom

transcripts determined in breast adipose tissue are assumed to be translated into P450arom protein with aromatase activity, which gives rise to local estrogen biosynthesis. At least two published studies from this laboratory support this assumption: Significant levels of aromatase activity have been demonstrated in the adipose tissue (3), and aromatase activity of adipose fibroblasts have been shown to be primarily regulated by changes in P450arom transcript levels (17).

One out of every nine American women will develop breast cancer. More than two-thirds of breast carcinomas develop in women above the age of 50. The present study provides data of an associative nature and does not necessarily demonstrate a cause and effect relationship between local estrogen biosynthesis and occurrence of breast cancer. However, it suggests that local factors in the breast may influence carcinogenesis. It has been hypothesized that 5 to 15 years are required for a single malignant cell to develop into a clinically detectable breast cancer tissue mass. One may then assume that conditions that predispose a postmenopausal woman to develop breast cancer may preeexist in her younger years. High local estrogen concentrations in the breast may predispose cancers initiated in these areas to develop more aggressively than in others. Breast cancer occurs in the outer quadrants in 71% of the cases in large series (18). The present study reveals that 64-67% of the highest P450arom transcript levels and adipose fibroblast content were also found in an outer breast region. On the other hand, we have previously shown in mastectomy specimens, that the highest transcript levels were detected in regions bearing tumors regardless of the tumor location (8). Tumors secrete products (19), *e.g.*, members of IL-6 cytokine subfamily, which stimulate aromatase expression in the surrounding adipose tissue (20). Moreover, estradiol stimulates cytokine secretion by T-47D breast cancer cells (our unpublished observations). These data collectively suggest that tumors preferentially develop in breast regions with the highest aromatase expression. Once developed, tumor growth is further promoted by high local estrogen concentrations which are maintained by the positive feedback relationship between adipose tissue aromatase expression and tumor-derived cytokines.

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FIGURE LEGENDS

Figure 1. Standard curve, revealing simple linear relationship between increasing amounts of total breast adipose tissue RNA and corresponding arbitrary units for P450arom transcript levels. ***Panel A:*** Coamplification of increasing amounts of breast adipose tissue total RNA and a constant quantity of rat P450arom cRNA internal standard (1 pg). Samples were amplified in duplicate. ***Panel B:*** A linear increase in breast P450arom levels (ratios of human to rat amplification products) was observed with increasing amounts of breast adipose tissue RNA. Statistical analysis was performed using simple linear regression with a coefficient of determination (r)=0.987 at a level of significance of α:0.05 and $p<0.0001$.

Figure 2. A representative slot blot autoradiograph from one patient is shown. We compared P450arom transcript levels in breast fat using 10 μg of total RNA from each quadrant. Each sample was assayed in duplicate (variation in duplicate samples ranged from 2 to 8%) and radioactivity on the blotting membranes was quantified by an AMBIS radioimaging system. A corrected AMBIS reading was calculated and recorded as an arbitrary unit for the P450arom transcript level for each quadrant as described in Figure 1.

Figure 3. **A.** A representative section of the breast adipose tissue with low fibroblast content. Whereas mature adipocytes (large cells with cytoplasms distended by lipid material and peripheral nuclei) constitute the majority of this section, a few scattered fibroblasts dispersed between mature adipocytes are seen. A very low P450arom mRNA level (normalized to total RNA content) was detected in this sample. **B.** A breast fat specimen showing higher numbers of fibroblasts. Fibroblasts are oval or spindle-shaped and do not contain conspicuous lipid in their cytoplasms. A markedly higher level of P450arom mRNA was found in this sample. (Hematoxylin and eosin stain, magnification: 200X. Note that handling and magnification for both sections are the same, whereas the size of

adipocytes varies.) Tissue components were quantified by computerized morphometry. A positive correlation was found between the tissue fibroblast content and P450arom transcript level in breast adipose tissue.

Table 1. Adipose Tissue P450arom Transcript Levels in Breast Regions.

Patient	Left			Right		
	Inner	Upper	Outer	Inner	Upper	Outer
1	0.21	0.28	0.12	0.21	0.10	0.07
2	1.31	1.80	2.23	1.23	2.47	1.74
3	0.13	0.13	0.18	0.11	0.14	0.22
4	2.76	2.80	2.29	3.85	1.98	2.23
5	1.01	0.80	0.33	1.58	1.24	2.54
6	0.07	0.03	0.08	0.05	0.01	0.07
7	3.67	18.40	2.80	4.60	7.60	4.20
8	0.96	1.60	1.01	1.07	0.77	1.90
9	1.30	4.60	4.70	2.30	3.30	4.84
10	1.25	3.78	2.03	3.46	3.40	3.08
11	1.20	1.89	1.03	1.87	1.31	1.95

Table 2. Fibroblast to Adipocyte Ratios in Breast Regions

Patient	Left			Right		
	Inner	Upper	Outer	Inner	Upper	Outer
1	0.03	0.15	0.12	0.09	0.05	0.05
2	0.02	0.06	0.10	0.04	0.05	0.06
3	0.12	0.11	0.13	0.04	0.07	0.11
4	0.12	0.02	0.05	0.12	0.04	0.06
5	0.06	0.04	0.09	0.10	0.10	0.05
6	0.05	0.03	0.11	0.04	0.03	0.06
7	0.05	0.15	0.05	0.06	0.09	0.15
8	0.10	0.06	0.08	0.09	0.05	0.12
9	0.03	0.04	0.01	0.02	0.02	0.07
10	0.03	0.05	0.06	0.10	0.04	0.10
12	0.05	0.04	0.06	0.07	0.10	0.12
13	0.04	0.03	0.10	0.07	0.05	0.11

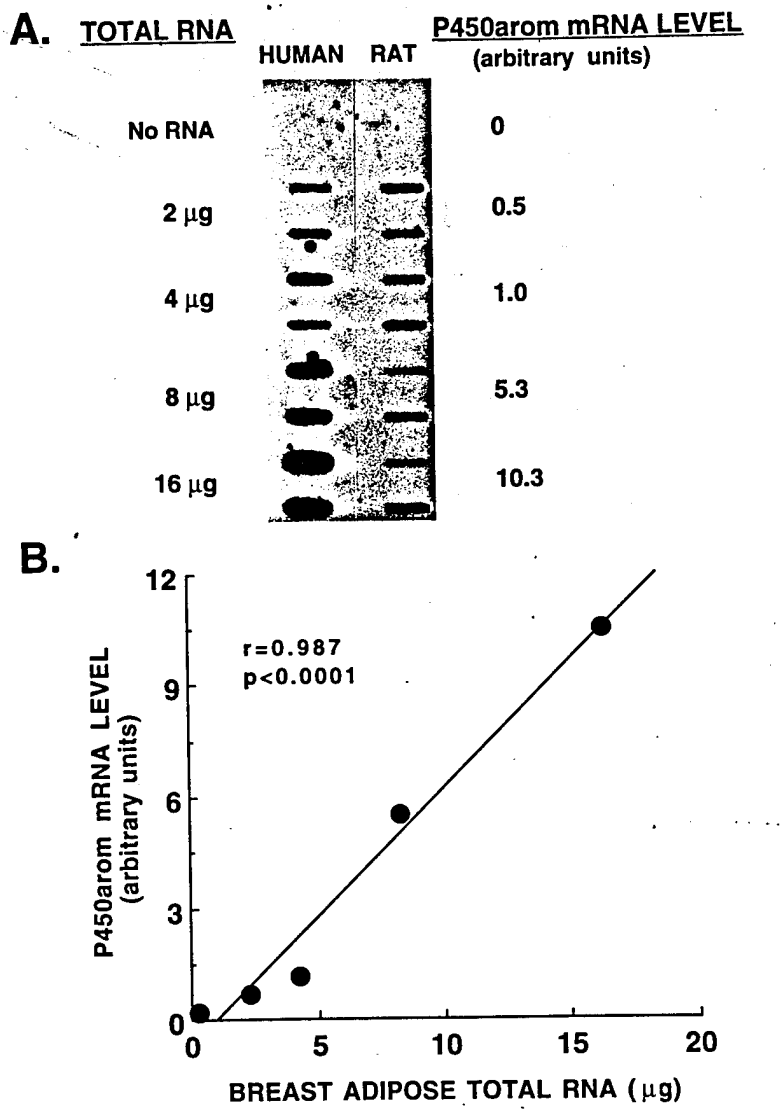
A.
TOTAL RNA
HUMAN
RAT
P450arom mRNA LEVEL
(arbitrary units)
No RNA
2 µg
4 µg
8 µg
16 µg
0
0.5
1.0
5.3
10.3
B.
r=0.987
p<0.0001
P450arom mRNA LEVEL
(arbitrary units)
12
9
6
3
0
0
5
10
15
20
BREAST ADIPOSE TOTAL RNA (µg)

FIGURE 1.

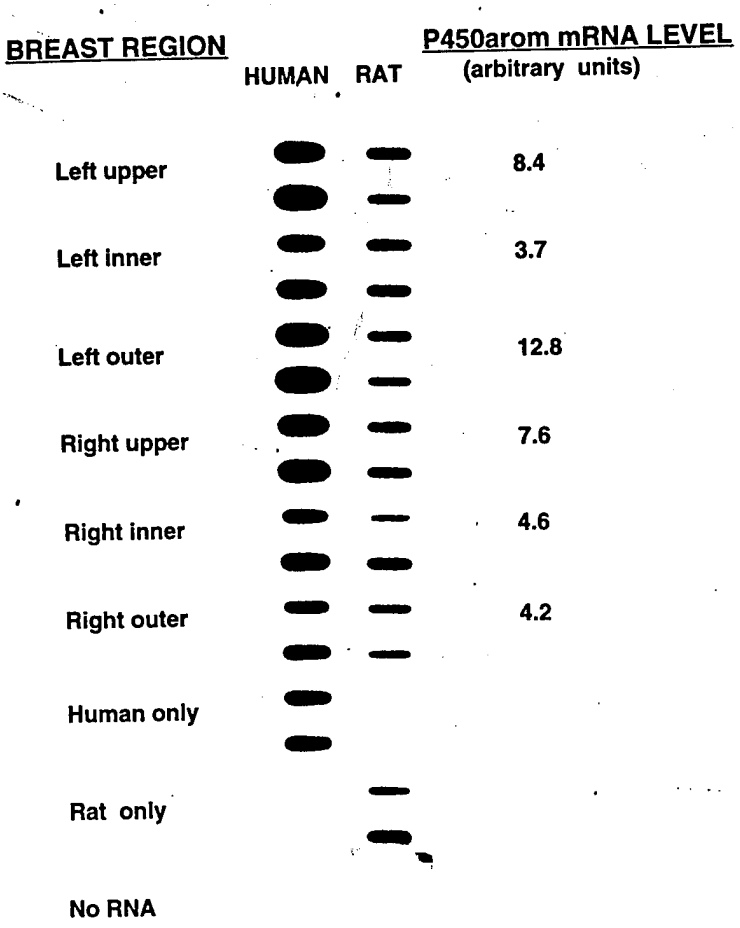
BREAST REGION
HUMAN
RAT
P450arom mRNA LEVEL
(arbitrary units)
Left upper
8.4
Left inner
3.7
Left outer
12.8
Right upper
7.6
Right inner
4.6
Right outer
4.2
Human only
Rat only
No RNA

FIGURE 2.

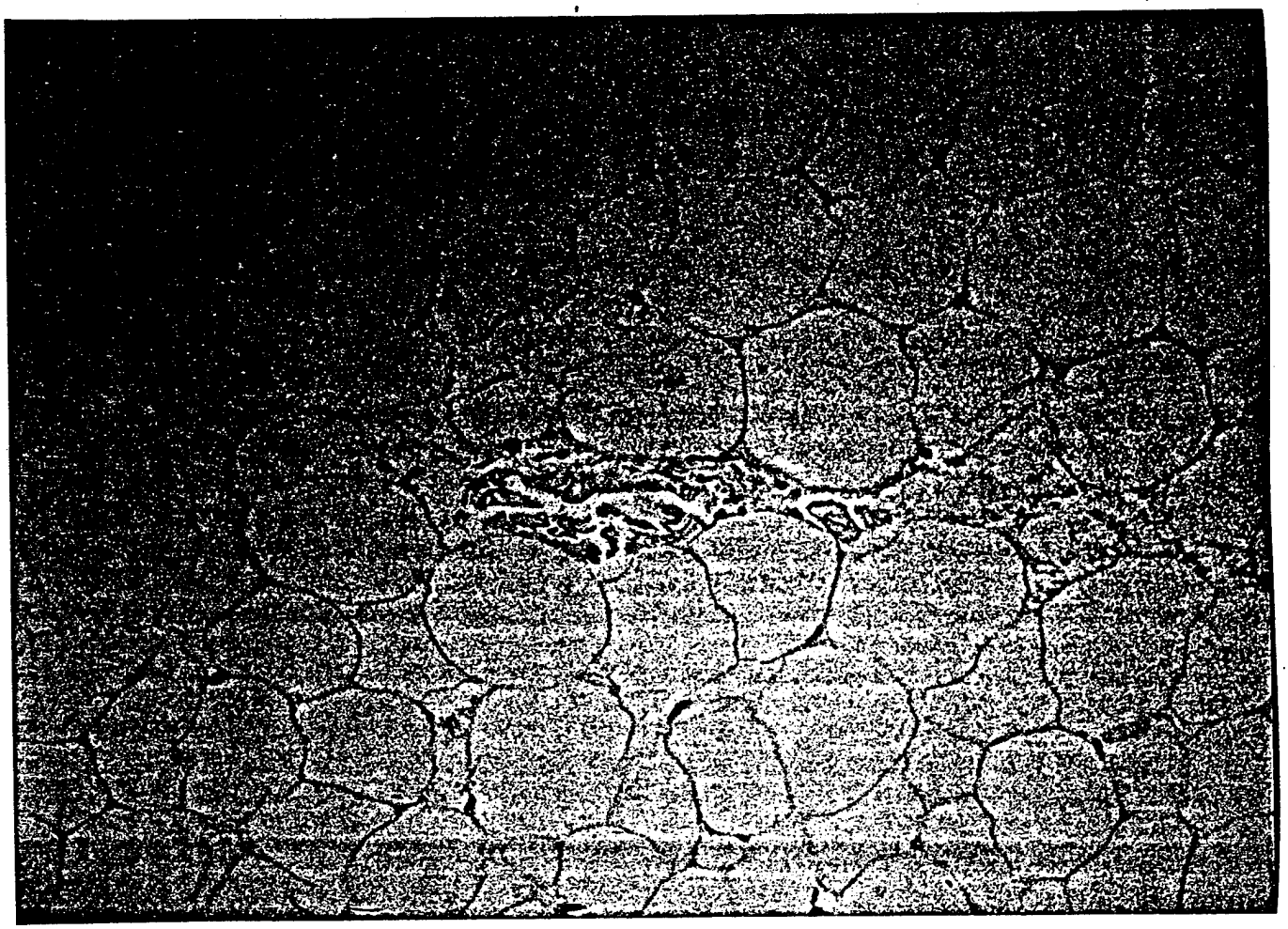

Figure 3a

Steroid measurements from adrenal veins, aromatase activity and aromatase P450 gene expression in a feminizing adrenocortical tumor.*

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Abstract

In a 29-yr-old man, gynecomastia and impotence were the clinical symptoms of an estrogen-secreting adrenocortical tumor. The diagnosis was made based upon high plasma estrone (E_1) and estradiol (E_2) levels and the finding of a 9 cm left adrenal mass on CT scan. Catheterization of adrenal veins showed markedly elevated estrone levels in the left adrenal venous effluent. Estradiol levels were not significantly different from the peripheral values indicating that practically all E_2 was formed from peripheral conversion of E_1. The increased plasma levels of estrogens were associated with a marked suppression of gonadotropin secretion. The absence of LH response to pulsatile GnRH administration contrasted with the rapid recovery of LH secretion in the postoperative period and suggested a direct action of estrogens at the pituitary level.

An extremely high level (214 pmol/mg protein/hour) of aromatase activity assayed by release of [^{3}H] water from [^{3}H] androstenedione was detected in microsomes of tumor tissue but not of normal adrenals. Accordingly, aromatase P450 (P450arom) transcripts in the tumor were detected using both Northern blot analysis and RT-PCR, whereas they could not be demonstrated in a normal adult adrenal using either method. In addition, in the adrenal tumor, the untranslated first exons in 5'-terminals of P450arom transcripts were studied. Only promoter II-specific transcripts were present, whereas exon I.3 and I.4-specific sequences were not detected. Therefore, promoter II, the gonadal type promoter was responsible for aromatase expression in this tumor.

The present study demonstrates for the first time that high aromatase activity in a feminizing adrenocortical carcinoma was mediated by abnormal expression of the P450arom gene in the adrenal tumoral tissue.

Adrenal androgens are substrates for estrogen production by peripheral tissues. However, it has not been demonstrated that estrogens could be synthesized by the normal adrenal (1). In contrast, adrenocortical carcinomas may produce directly large amounts of estrogens (2-4). These estrogen secreting tumors are extremely rare (2-11).

In the present report, a patient with gynecomastia was found to have such an adrenocortical carcinoma. This case provided the opportunity for appraising the mechanisms involved in abnormal estrogen biosynthesis. Estrogen production was evaluated *in vivo* by direct sampling from adrenal veins and *in vitro* by measurement of aromatase activity in the tumoral tissue. Expression of the aromatase P450 (P450arom) gene was studied in this tumor comparatively to normal adrenals. In addition, the untranslated first exons in 5'terminals of P450arom transcripts were studied.

A recent report has emphasized the role of estrogens on gonadotropin secretion in men (12). Thus, it was of interest to further study the nature of endogenous estrogen action on the hypothalamo-pituitary gonadal axis in men.

Case report

A 29-year-old man was referred for evaluation of progressive bilateral gynecomastia, diminished libido, and impotence. There was no history of exposure to exogenous estrogen, androgen antagonists or alcohol. Physical examination revealed symmetric gynecomastia (5 by 5 cm), a feminized body contour and diminished testicular size (9 and 8 ml). There were no clinical features suggestive of cortisol excess. Blood pressure was normal (130/85 mm Hg). Electrolytes, glucose, liver and thyroid functions were normal. The sperm count showed azoospermia. A computerized axial tomographic scan of the abdomen revealed a left adrenal mass (9x9x10 cm). After detailed endocrine preoperative testing and catheterization of adrenal veins the patient underwent left adrenalectomy. An adrenal cortical carcinoma was removed without complications. Part of it was immediately frozen in liquid nitrogen for biochemical and molecular studies. Histopathological examination of the resected tumor showed a large encapsulated tumor. The weight of the tumor was 210 g. The cut surface appeared pale brown in color, zones of necrosis and haemorrhage

were observed. On microscopic examination, a diffuse proliferation of polygonal cells arranged in trabecular form was observed. Severe nuclear pleomorphism was present, with frequent mitoses. Extensive areas of confluent necrosis were present and multiple foci of vascular invasion were seen. After resection of the tumor, plasma estrone (E_1) and estradiol (E_2) levels fell dramatically. Three months later, gynecomastia had regressed and the patient had normal sexual intercourses. Four years after surgery, whereas E_1 and E_2 remained in the normal range, a chest CT scan detected a lung nodule. The resection and histological analysis of this nodule confirmed the existence of a distant metastasis and mitotane therapy was started.

Material and methods

Catheterization

Before surgery, catheterization was performed at 0800 h. Two catheters were introduced through the femoral vein under radiological control into the left and right adrenal veins. A third catheter was placed in a peripheral vein of the forearm. Therefore, sampling in the three veins could be performed simultaneously and three samples were taken at 30-min intervals. Six men with an incidentally discovered non hormone-secreting adrenocortical tumor were used as controls.

Hormone assays

Plasma E_1 and E_2 concentrations were measured in duplicate by specific radioimmunoassays as previously described (13). The following steroids were measured by radioimmunoassay after chromatographic separation on a sephadex LH 20 or celite column as previously described (14-16) : dehydroepiandrosterone (DHEA) and its sulfate ester DHEAS, progesterone (P), desoxycorticosterone (DOC), aldosterone, 17-hydroxyprogesterone (17-OHP), androstenedione (A), testosterone (T), desoxycortisol (S) and cortisol (F). Inter and intraassay precision coefficients of variation for these plasma steroid RIAs were less than 10 % and 15 % respectively. The intraassay and interassay coefficients of variation were 1.5 % and 5.2 % respectively. ACTH was measured by immunoradiometric assay with an immunoassay kit (Allegro HS-ACTH) purchased from Nichols Institute (San Juan Capristano, CA). Plasma immunoreactive FSH and LH concentrations were measured in duplicate by means of a monoclonal antibody immunoradiometric assay (IRMA) as previously described (17).

Study of pulsatile LH secretion

Before surgery, LH secretion was evaluated in basal condition and ten days after pulsatile GnRH administration at a dose of 20 µg/pulse sc every 60 min for 5 h. Blood sampling was initiated at 0800 h and sampled every 10 min for 5 h. In the postoperative period (9 days after surgery) LH secretion was reevaluated.

Aromatase activity

Normal human adrenal tissue was obtained at the time of nephrectomy performed on three patients for the treatment of kidney cancer. Aromatase activity was measured in microsomes prepared from normal adrenal tissue, the tumor and human placenta (positive controls) (18). Proteins were measured by the Bradford technique (19) using serum albumin as a standard. Total aromatase activity was measured by the tritiated water technique with 0.7 µM [1β-^{3}H]androstenedione (obtained from NEN/Dupont) as substrate as previously described (20). At the end of the incubation, tritiated steroids were removed from the medium by chloroform extraction followed by dextran-charcoal adsorption. The remaining radioactivity, representing the tritiated water generated during the aromatization was measured. Results were expressed as the amount of aromatized precursor, calculated from the amount of tritiated water formed. When a sample of [1β-^{3}H]androstenedione was completely aromatized by human placental microsomes, only 65% of the label was released as tritiated water (results not shown). The data were corrected accordingly.

RNA blot hybridization analysis

Total RNA isolation.

Total RNA was isolated from frozen tissue by the guanidinium thiocyanate-cesium chloride method. RNA concentration was determined by spectrophotometric absorption at 260 nm.

Northern analysis.

Total RNA was size-fractionated by electrophoresis on formaldehyde-agarose gel and transferred to a nylon membrane. Hybridization was conducted using an assymmetric PCR-generated single-stranded complementary DNA (cDNA) probe radiolabelled with [^{32}P]deoxycytidine triphosphate (dCTP). The first two primers in Table 1 were used to generate a 195 bp-long probe

complementary to the coding region of P450arom cDNA. The antisense primer was added in excess.

Polymerase chain reaction after reverse transcription (RT-PCR).

Total RNA was isolated from adrenal tumor and normal disease-free adrenals. RNA was initially treated with DNase I to remove any contaminating DNA. Total RNA (300 ng) was then reverse transcribed using random hexamers (Gibco-BRL). cDNA was amplified for 25 cycles. The primers used for the amplification are listed in Table 1. An antisense primer complementary to exon III was used in all reactions. Sense oligonucleotides specific for the unique untranslated 5'-ends of P450arom transcripts were used to amplify the specific sequences of known length. A trace amount of [^{32}P]dCTP was added to each of the PCR reactions. The reaction products were analysed on 4% non denaturing polyacrylamide gels. Radioactive gels were exposed to X-ray film.
Transcripts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, were separately amplified by RT-PCR to check the integrity and comparative quantity of total RNA and cDNA used in amplification of P450arom transcripts. Primers targeted to amplify the coding region of GAPDH cDNA are listed in Table 1.

Results

Free urinary cortisol and plasma hormones in peripheral veins

Free urinary cortisol, measured at three different days, was not increased (range:121-223 nmol/24h ; normal 54-243 nmol/24h). Plasma hormone levels in a peripheral vein before and after surgery are shown in Table 2. Basal cortisol at 0800 h was in the normal range but the diurnal variation was abolished (data not shown). However, basal plasma ACTH levels were low and did not increase after a CRH test (100 µg IV). Plasma 17-OHP was in the normal range. Plasma S level was increased but plasma DOC level was normal. Aldosterone (Table 2) and plasma active renin (not shown) were in the normal range. Plasma A concentration was in the normal range whereas plasma DHEA and DHEAS levels were low. Plasma testosterone levels were low. Before surgery, circulating E_1 and E_2 levels were 10- to 20-times higher than those in normal subjects. E_1/E_2 ratio was 1.5. In the postoperative period, plasma E_1, E_2 and S levels decreased dramatically, F remained in the

normal range but plasma ACTH increased. The adrenal androgens DHEA, DHEAS and A were low.

Steroids in adrenal veins

Plasma steroid levels in the adrenal veins are shown in Table 3. On the tumor side, plasma E_1 levels were very high (8 fold greater than in the peripheral vein). E_1/E_2 ratio was 13.9. Plasma E_2 levels were elevated but in contrast to plasma E_1, in the same order of magnitude as in peripheral blood. T was in the normal range. It is noteworthy that, contrary to the normal adrenal plasma A, DHEA and DHEAS levels were low. The normal plasma F level demonstrated that the tumor was the source of this steroid. Plasma S level greater in the adrenal vein than in the peripheral vein suggested a partial 11β hydroxylase deficiency in the tumor. However, plasma DOC levels were not increased. Plasma aldosterone level was extremely low indicating the destruction of the glomerulosa zone by the tumor. Plasma 17OHP level was in the normal range.
In the right adrenal vein, the elevated aldosterone levels confirmed the correct position of the catheter in this vein. Plasma E_1 and E_2 levels were not different from those in the peripheral vein. The concentrations of plasma F, S, T, A, DHEA, DHEAS and 17OHP in this venous effluent were low and not different from those observed in the peripheral blood.

Gonadotropin secretion

Results are shown in Fig. 1. In the preoperative period LH and FSH were almost undetectable (0.5 UI/L and 0.4 UI respectively) and did not increase after GnRH injection (100 μg, iv). Pulsatile LH secretion was suppressed. Pulsatile GnRH administration failed to induce a normal pattern of endogenous LH secretion and did not restore gonadotropin response to the GnRH test. In contrast, nine days after surgery, pulsatile LH profile was restored with a frequency of 1 pulse per hour and a normal response of both gonadotropins to the GnRH test was observed.

Aromatase activity

Aromatase microsomal activities in three normal human adrenals, the adrenal tumor and in human placenta are presented in Fig. 2. Aromatase activity was not detected in normal adrenals. In contrast, a very high level of aromatase activity was observed in the microsomes of the tumor tissue [214 ± 90 pmol/mg protein.h (mean ± SD)]. In human placental microsomes (used as positive

control), aromatase activity was 7340 ± 1230 pmol/mg prot.h (mean ± SD, n = 3 differents samples).

Northern analysis

Total RNA samples from adrenal tumor (Fig.3 lane 2), a disease-free normal adult adrenal (lane 3), and three control samples including fetal liver (lane 1), placenta (lane 4) and cAMP-treated adipose fibroblasts in culture (lane 5) were hybridized with a 195 bp-long cDNA probe complementary to coding region of P450arom mRNA. The lower panel of Fig. 3 shows the comparative quantity and quality of the RNA samples. In the upper panel of Fig. 3, P450 arom mRNA species of expected size (3.4 kb and 2.9 kb) were detected in adrenal tumor sample (lane 2), whereas no P450arom transcripts were detected in normal adrenal (lane 3), as expected.

P450arom promoter usage

RT-PCR analysis was performed by using RNA from adrenal tumor and normal adrenal. In the adrenal tumor, promoter II-specific transcripts were present (Fig. 4A, left panel, second lane), whereas exon I.3- and I.4-specific sequences were not detected. The placental untranslated exon I.1-specific sequences also could not be amplified using PCR (data not shown). The region of P450arom cDNA flanking coding exons II and III was amplified as a control for the total amount of P450arom transcripts. The intensity of the bands corresponding to promoter II and coding region was similar confirming that all P450arom transcripts in this adrenal tumor contained only promoter II-specific sequence (Fig. 4A, left panel, first lane). Therefore, it follows that the gonadal type promoter II was responsible for aromatase expression in this adrenal tumor whereas the adipose-type (I-3 and I-4) or placental- type (I-1) promoters were not utilized. As shown in right panel of Fig. 4A, P450arom transcripts were not detected in the normal adrenal sample. This confirms the result of northern analysis. To check the quality and quantity of total RNA and cDNA synthetized, amplification of an endogenous marker, GAPDH transcripts, was performed (Fig. 4B). Separate amplification of GAPDH transcripts revealed presence of intact RNA of comparable amounts (Fig. 4B) in both the adrenal tumor and disease-free adrenal samples. The size of each amplified band was consistent with that of the expected size, e.g. P450arom coding region-195bp, P450arom promoter II-specific sequence-305bp and GAPDH-306bp.
Total RNA from dexamethasone- and dibutyryl cyclic AMP-treated adipose fibroblasts were used as positive controls for exon I-3 and I-4-specific sequences, respectively (data not shown). Total RNA

from human endometrium was used as a negative control (data not shown).

Discussion

The present study reports the uncommon discovery of a feminizing adrenocortical carcinoma in a patient with gynecomastia (21). These tumors are rare and fewer than 100 cases have been reported (2-11). In the absence of absolute clinical, biological, anatomical or histological criteria, it is well known that in many cases the benign or malignant nature of an adrenal tumor cannot be strictly asserted. However, in the present case, high plasma levels of estrogens, severe nuclear pleomorphism, mitoses and foci of vascular invasion suggested its malignant nature. Finally, the existence of a distant metastasis confirmed the diagnosis of malignant adrenal carcinoma.

Free urinary and plasma F levels in the left adrenal vein were normal. However, the autonomous glucocorticoid secretion by the tumor was supported by the absence of diurnal variation of plasma F associated with low basal and CRH-stimulated plasma ACTH levels. The ACTH insufficiency was further supported by similar plasma levels of F and adrenal androgens in the right adrenal and peripheral veins. In addition, increased baseline S levels in the peripheral veins and the left adrenal vein indicated a partial 11β hydroxylase deficiency as previously reported (2-4).

The main characteristic of this tumor was its ability to produce estrogens. In this patient, circulating E_1 and E_2 levels were 10 to 20 times higher than that in normal subjects. Adrenal venous sampling confirmed the left adrenal mass to be the source of E_1 and its precursor A. This result suggested the presence of an aromatase activity within the tumor and excluded the possibility of an extra adrenal aromatization of A as a source of estrogen excess. The enzymatic study performed in the tumoral tissue confirmed this hypothesis. A very high level of aromatase activity was demonstrated in the tumor, whereas aromatase activity was not detected in normal adrenals. Although, tumoral aromatase activity was 34 times less than that in placenta, this was still an extremely high level. For example, it was 200 times higher than aromatase activity of the adipose tissue (20). Taken together, *in vivo* and *in vitro* data showed that the tumoral adrenal had the capacity to synthetize E_1 very efficiently. Whereas E_1 from the left adrenal vein was 8 times higher than the peripheral levels, E_2 levels were not significantly different from the peripheral values. Thus, it appears that almost all E_2 production originated from peripheral conversion of E_1 but not from adrenal secretion. The E_1/

E_2 ratio markedly higher in the left adrenal than in peripheral blood suggested similarly the conversion of the weak estrogen E_1, secreted by the tumor, into the active estrogen E_2 by an extraadrenal 17β-hydroxysteroid dehydrogenase (22-24).

P450arom catalyzes conversion of C19 steroids into estrogens in a number of human tissues such as the ovary, testis, placenta, adipose tissue and the brain (25). Aromatase expression has also been detected in several tumors such as the ovarian granulosa cell tumor, testicular Sertoli cell tumor (26), endometrial carcinoma (27), uterine leiomyoma (28) and hepatocellular carcinoma (29). In this report, we present evidence for massive aromatization of C19 steroids by an adrenocortical tumor. Aromatase expression in human tissues is regulated in part by alternative use of tissue specific promoters (25). The distal promoter I.1 is exclusively used in placenta, whereas promoters I.3 and I.4 are responsible for aromatase expression in adipose tissue (25). I.4 is also used in fetal liver and skin fibroblasts (25). The classically located proximal promoter II directs P450arom gene transcription in the gonads (26). In adipose fibroblasts in culture, switching of promoter use between promoter II, I.3 and I.4 was observed under various hormonal conditions (30). Incubation of adipose fibroblasts with cyclic (c) AMP analogs in the absence of serum induced aromatase expression by promoter II and I.3 (25). The present sudy demonstrates that promoter II is responsible for aromatase expression in an adrenocortical tumor, as previously shown in various tumors (26-29). Whereas this is not surprising in gonadal tumors, it is intriguing to consistently observe use of promoter II in the other tumors, because disease-free tissues of origin, namely normal endometrium, myometrium, adult liver and adult adrenal, do not express aromatase (28, 31, 32). Activation of a common cAMP-related signalling pathway may be responsible for initiation of transcription via promoter II in these tumors.

It has been shown that T is a major component in the feed-back mechanism governing pulsatile LH secretion in man by acting at the hypothalamic level (33). However, T may also inhibit gonadotropin secretion after aromatization to E2 (34, 35). The importance of estrogens in the regulation of the gonadotrope axis in men has been suggested in patients exposed to exogenous estrogens (36) and demonstrated in subjects treated with antiestrogen or aromatase inhibitors (37, 38). More recently it has been reported in a man with estrogen resistance caused by a mutation in the estrogen-receptor gene that gonadotropins were increased despite normal testosterone levels (12). In the present report, sustained endogenous estrogen excess verifies these previous data and specifies the site of action of estrogens on gonadotropin secretion. Before surgery, the increased serum concentration of E_1 and E_2

were associated with a marked decrease of plasma LH and FSH with severe androgen deficiency and azoospermia. The absence of LH response to pulsatile GnRH administration contrasted with the rapid recovery of LH and FSH secretion after suppression of estrogen excess. These data strongly suggest that estrogen acted directly on the pituitary to decrease gonadotropin secretion.

In summary, the present study demonstrated for the first time an abnormal expression of the CYP19 (P450arom) gene responsible for the very high aromatase activity in a feminizing adrenocortical carcinoma. Only promoter II, the gonadal type, was responsible for aromatase expression in this tumor. In addition, the high plasma levels of estrogens were associated with a marked suppression of gonadotropin secretion at the pituitary level.

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Legends

Figure 1. Gonadotropin secretion before and ten days after pulsatile exogenous GnRH administration in the preoperative period and 9 days after removal of the estrogen-secreting adrenal tumor.
A Basal and GnRH (100μg IV) induced peak levels of gonadotropins.
B LH pulsatile profiles. Plasma E2 (pmol/L) and T levels (nmol/L)

Figure 2. Microsomal aromatase activity in three normal human adrenals in the adrenal tumor and in human placenta. Values are expressed as the mean ± sd (see methods). Note the log scale. ND = Not detectable.

Figure 3. UPPER PANEL : Northern blot analysis revealing presence of P450arom transcripts in adrenal tumor RNA sample (lane 2). Two bands of expected sizes (3.4 kb and 2.9 kb) are noted. No P450arom transcripts were detected in disease-free adrenal (lane 3). Readily hybridizable P450arom transcripts were detected in control tissue samples : fetal liver (lane 1), placenta (lane 4) and cAMP-treated adipose fibroblasts (lane 5). LOWER PANEL : Ethidium bromide-stained 28S (upper) and 18S (lower) bands are demonstrated in the same RNA samples before transfer to a nylon membrane. Total RNA was used in the first 3 lanes (20μg) and the fourth lane (10μg). Poly A^+RNA (0.5μg) was used in lane 5. First three samples were exposed to an autoradiograph for 72h, whereas the exposure period is 2h for the last two samples due to abundance of P450arom transcripts in placenta and cAMP-treated adipose fibroblasts.

Figure 4. **A** : After reverse transcription, P450arom cDNA samples were amplified using primers specific for unique untranslated 5'ends of P450arom transcripts. Only promoter II specific sequence could be amplified in adrenal tumor (left panel, second lane), whereas promoter I.3- and I.4-specific transcripts were not detected (lanes 3 and 4). As a control for total P450arom transcript level, a coding region that is common to all P450arom transcripts was amplified (first lane), and bands for promoter II-specific sequence and common coding region were of similar intensity indicating that only promoter II was responsible for aromatase expression in the adrenal tumor. No P450arom transcripts could be amplified in disease-free adrenal sample (right panel) confirming the results of northern blot analysis.
B : GADPH transcripts were amplified in total RNA samples to confirm use of comparable amounts of intact RNA.

Table 1 : Primers used for PCR amplification of 5' -untranslated ends and coding region of P450arom transcripts and coding region of GAPDH transcripts.

3' antisense primer for coding exon III
5' CAG GAA TCT GCC GTG GGG AT 3'

5' sense primer for coding exon II
5' TTG GAA ATG CTG AAC CCG AT 3'

5' sense primer for untranslated exon I.4
5' GTG ACC AAC TGG AGC CTG 3'

5' sense primer for untranslated exon 1.3
5' GAT AAG GTT CTA TCA GAC C 3'

5' sense primer for promoter II-specific sequence
5' GCA ACA GGA GCT ATA GAT 3'

5' sense primer for untranslated exon I.1
5' GAA CAC GTG GAG GCA AAC 3'

GAPDH1 : 5' sense primer for GAPDH cDNA
5' CGG AGT CAA CGG ATT TGG TCG TAT 3'

GAPDH2 : 3' antisense primer for GAPDH cDNA
5' AGC CTT CTC CAT GGT GGT GAA GAC 3'

Table 2. Plasma hormones levels in a peripheral vein before and five days after tumor resection (nmoles/L, *pmoles/L)

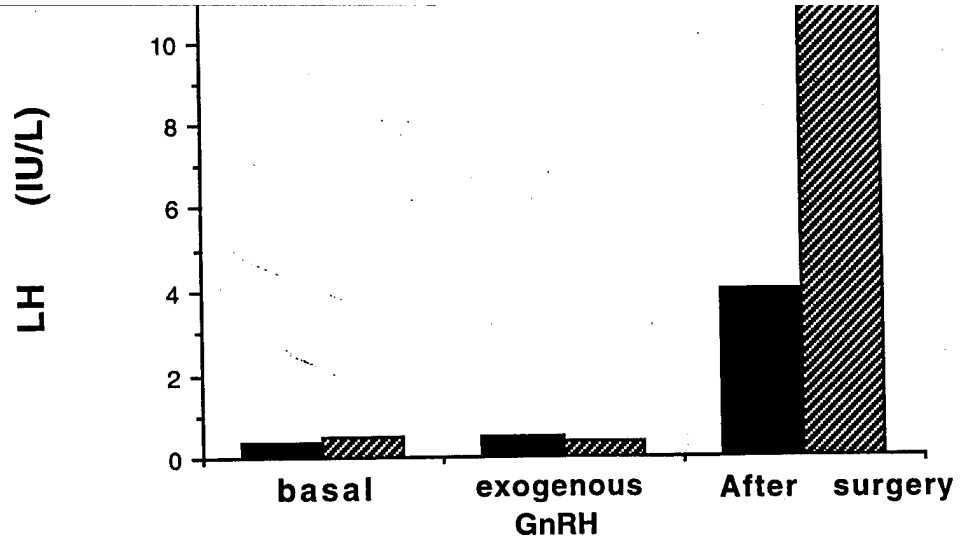
	Before surgery	After surgery	Normal range
F	548	312	250-650
ACTH*	1.1	10.2	4.5-10
17-OHP	5.8	0.6	3.0-8.0
S	70	1.2	0-3.0
DOC	0.17	0.21	0.12-0.5
Aldosterone	0.15	0.12	0.09-0.3
A	4.9	1.9	2.8-4.9
DHEA	2.0	1.5	3.5-38
DHEAS	1,292	952	3,800-14,000
T	2.4	3.5	6.0-10.0
E1*	2,790	40	100-280
E2*	1,890	70	50-130

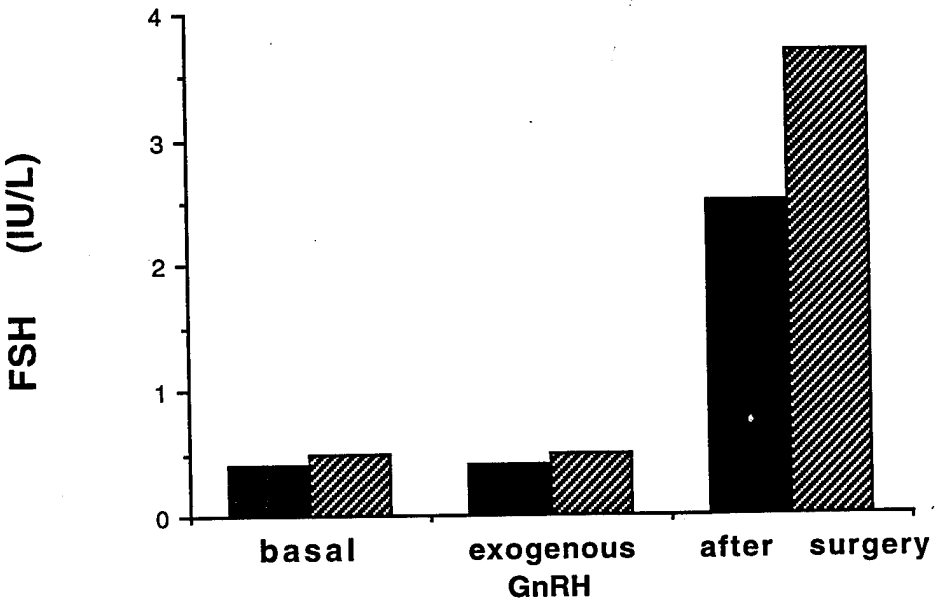
Data represent mean values of three pooled samples taken at 30-min intervals.

Table 3. Plasma steroid levels in the adrenal veins (nmoles/L, *pmoles/L)

	Left adrenal vein (Tumor)	Right adrenal vein	Normal range[a]
E1*	20,900	2,700	200-400
E2*	1,500	1,560	0-150
T	7.8	4.3	3.0-12.0
A	184	3.3	52-109
DHEA	15.2	4.0	2198-2884
DHEAS	2,508	2,343	1,2420-25,313
F	2,430	648	2,300-4,500
S	176	54	
DOC	27.9	ND	12-87
Aldosterone	0.15	13.9	7.3-15.4
17-OHP	15.0	4.0	14-29

Data represent mean values of three pooled samples taken at 30-min intervals. ND = not done. [a]Hormone levels in six men with a nonhormone secreting adrenocortical tumor (personal data, ref 15)


LH (IU/L)
10
8
6
4
2
0
basal
exogenous GnRH
After surgery


FSH (IU/L)
4
3
2
1
0
basal
exogenous GnRH
after surgery

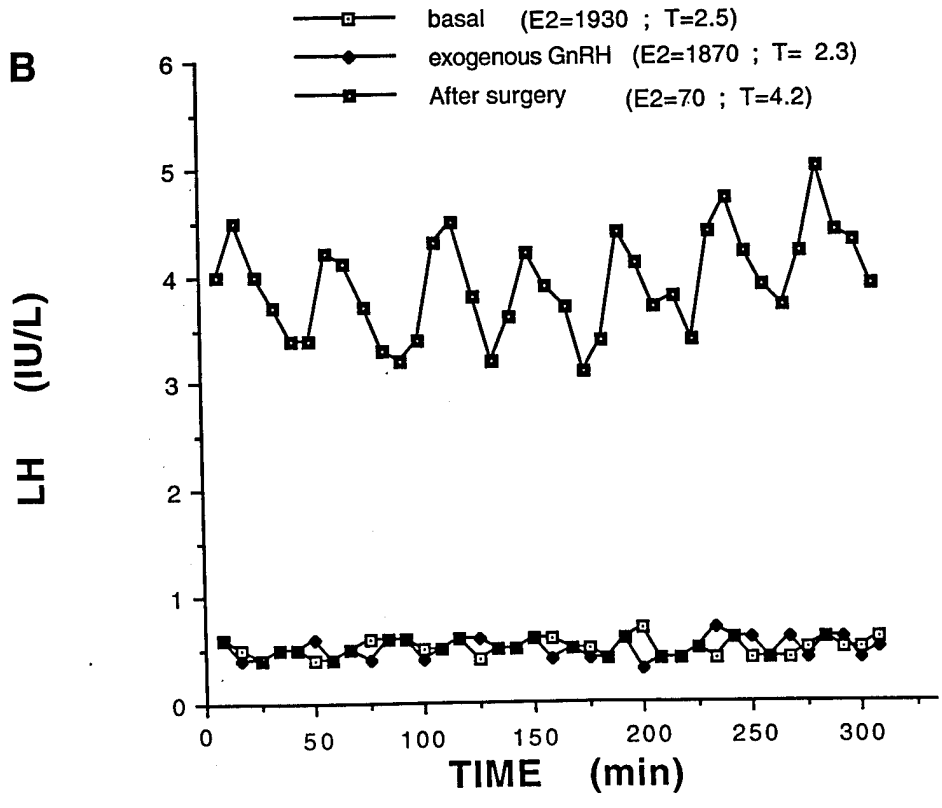

B
basal (E2=1930 ; T=2.5)
exogenous GnRH (E2=1870 ; T= 2.3)
After surgery (E2=70 ; T=4.2)
LH (IU/L)
6
5
4
3
2
1
0
0
50
100
150
200
250
300
TIME (min)

Figure 1

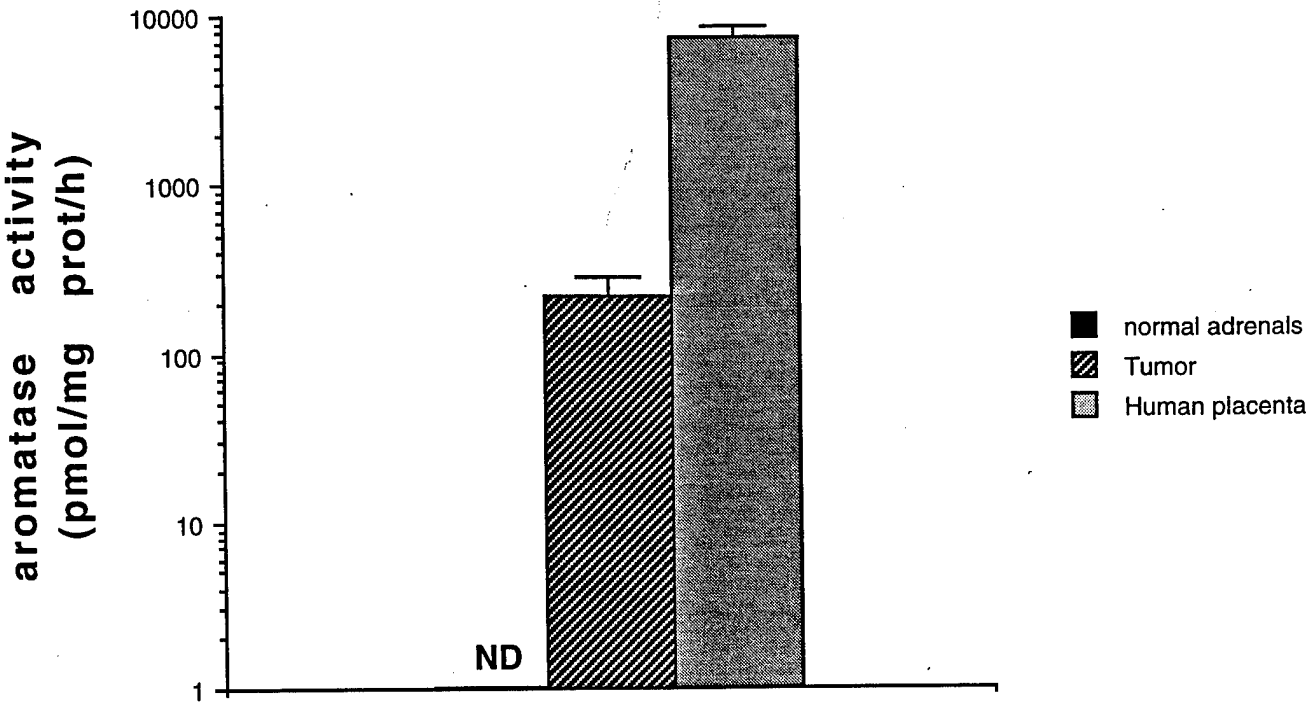
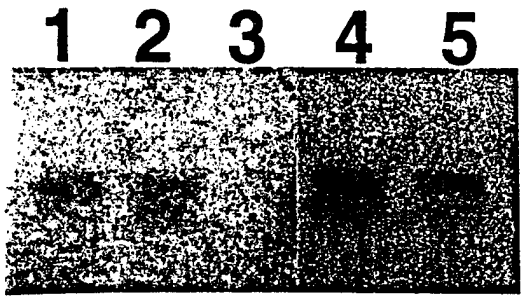

aromatase activity
(pmol/mg prot/h)
10000
1000
100
10
1
ND
normal adrenals
Tumor
Human placenta

Figure 2



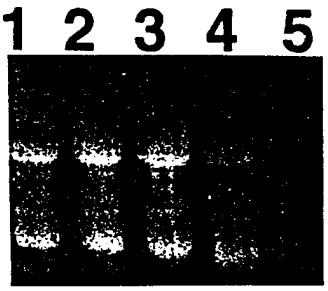
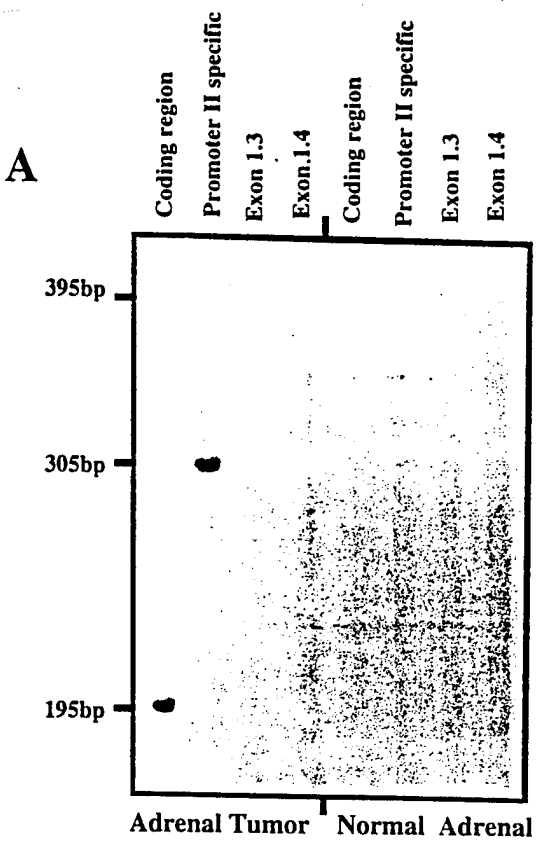


Figure 3


A
Coding region
Promoter II specific
Exon 1.3
Exon.1.4
Coding region
Promoter II specific
Exon 1.3
Exon 1.4
395bp
305bp
195bp
Adrenal Tumor
Normal Adrenal

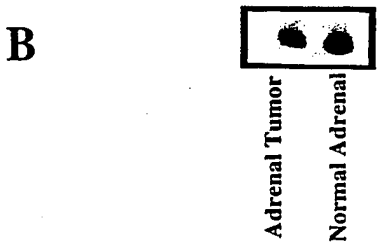

B
Adrenal Tumor
Normal Adrenal

Figure 4

Effects of Conditioned Medium From Different Cultured Cell Types on Aromatase Expression in Adipose Stromal Cells

John E. Nichols, MD, Serdar E. Bulun, MD, and Evan R. Simpson, PhD

OBJECTIVE: *To determine whether serum-free (SF) conditioned media (CM) from several human breast cancer cell lines and primary stromal cell cultures contain factor(s) that mimic the marked stimulatory effects of serum on aromatase activity and aromatase P450 (P450arom) gene expression in adipose stromal cells in culture (ASC) in the presence of dexamethasone (DEX).*

METHODS: *Adipose stromal cells, harvested from fresh adipose specimens, were grown to confluence, switched to SF media, and then incubated in the presence or absence of DEX with CM from T47-D breast cancer cells, pre-treated with or without 17β-estradiol (E2), and with CM from stromal cell cultures. Aromatase activity of the ASC was determined by the [^{3}H]water release assay. Total RNA was isolated, and reverse transcription-polymerase chain reaction was performed to determine the expression of various 5′-termini.*

RESULTS: *T47-D CM stimulated aromatase activity in a concentration-dependent manner, similar to that of serum, in ASC incubated with DEX. Estrogen potentiated this in a dose-dependent fashion. The ASC CM and endometrial stromal cell CM also markedly induced aromatase activity in ASC. Heat inactivation destroyed the stimulating ability of CM. The majority of P450arom 5′-termini expressed by ASC incubated with CM plus DEX contained the promoter I.4-specific sequence.*

CONCLUSIONS: *Conditioned media from several breast cancer cell lines and primary stromal cell cultures can mimic the effects of serum in the presence of DEX to stimulate aromatase activity in ASC. These results suggest that undefined, heat-labile and proteinaceous factors are present in CM that stimulate P450arom expression in a fashion similar to that of serum.* (J Soc Gynecol Invest 1995;2: 45–50)

KEY WORDS: Aromatase, adipose, conditioned medium, breast cancer.

In humans, estrogen biosynthesis occurs in a number of cells and tissues, including the granulosa cells of the ovary,[1] Leydig cells of the testis,[1] syncytiotrophoblast of the placenta,[2] various sites in the brain,[3,4] and in adipose tissue.[5] Conversion of C-19 steroids to estrogens is catalyzed by aromatase P450 (P450arom, the product of the CYP19 gene) in conjunction with the ubiquitous flavoprotein, NADPH-P450 reductase.[6,7] Adipose tissue is the major extragonadal site of estrogen production in both men and women.[8] Adipose tissue estrogen biosynthesis increases not only as a function of body weight and age,[9–11] but there also appears to be a marked increase in the ratio of breast adipose to breast glandular tissue with aging. Thus, in postmenopausal women, adipose tissue is the principal site of estrogen biosynthesis and has been correlated with the incidence of both endometrial and breast cancer.[12,13] Indeed, women with central obesity have up to three times the risk of developing breast cancer.[14] O'Neill et al[15] and Bulun et al[13] independently presented data indicating higher aromatase activity and expression in tumor-containing breast quadrants as compared to non-tumor-containing breast quadrants. It is well known that certain breast tumors can undergo marked cellular proliferation and production of local growth factors in the presence of estrogens, and, in turn, these tumor-secreted growth factors may induce surrounding adipose tissue to increase aromatase activity further by increased expression of the P450arom gene.[16]

Recent studies have indicated that tissue-specific expression of the aromatase CYP19 gene is regulated, at

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least in part, through use of tissue-specific promoters by means of alternative splicing mechanisms.[17–20] Aromatase expression in the ovaries and testes is driven by a proximal promoter (PII), which is located just upstream from the start site of translation. On the other hand, expression in the placenta is driven primarily from a distal promoter, I.1, located at least 40 kb upstream from the start of transcription. Furthermore, expression in adipose tissue appears to be regulated by use of yet another promoter, I.4, located 20 kb downstream from the placental promoter I.1.[17] Untranslated exons containing the starts of transcription from each of these tissue-specific promoters are spliced into a common 3′ splice junction just upstream from the start of translation in exon II of the P450arom gene. Thus, transcripts in these different tissue-specific sites of expression contain unique 5′ termini; however, the coding region and, therefore, the protein products are identical in each tissue site of expression.

As a consequence of the use of alternative promoters in this fashion, regulatory mechanisms controlling the expression of aromatase in each of the tissue sites of expression are quite different. In adipose stromal cells (ASC), aromatase expression is stimulated modestly by glucocorticoids, giving rise to P450arom transcripts with 5′ ends specific for promoter I.4.[17] However, with the addition of serum to glucocorticoid-treated ASC, aromatase activity is markedly increased.[21] This is because flanking sequences upstream from promoter I.4 of the CYP19 gene contain elements that respond to both glucocorticoids and factor(s) in serum.[22] To begin to understand which factor(s) might be responsible for mediating the stimulation of aromatase expression in ASC, we sought to determine whether the effect of serum can be mimicked by conditioned medium (CM) from a number of cells in culture, including several epithelial breast cancer cell lines such as MCF-7 and T47-D cells, endometrial stromal cells in culture, as well as CM from ASC themselves. We found that CM from each of these cells in culture was able to mimic the effect of serum to stimulate aromatase expression by ASC.

MATERIALS AND METHODS

Tissue Acquisition and Processing

Subcutaneous adipose tissue was obtained at the time of surgery from women undergoing breast reduction mammoplasty, abdominoplasty, or liposuction for either symptomatic macromastia or obesity. Consent forms and protocols were approved by the Institutional Review Board, University of Texas Southwestern Medical Center, Dallas. Adipose tissue was minced finely and incubated in Krebs bicarbonate buffer containing bovine serum albumin (4% wt/vol) (Pentax fraction V; Miles Laboratories, Elkhart, IN) and collagenase (0.6 mg/mL) (type 1A; Sigma, St. Louis, MO) for 45 minutes in a 37°C shaking water bath. After incubation, the undigested tissue was removed by filtering through a gauze mesh, and the resulting preparation was centrifuged at 400 × *g* for 7 minutes. The floating adipocyte and lipid layer was removed, and the remaining stromal cell pellet was washed and centrifuged twice more to remove any remaining collagenase.

Cell Cultures

Adipose stromal cells were suspended in Waymouth's MB 752/1 enriched media (Gibco/BRL, Grand Island, NY) containing 10% NuSerum (Collaborative Research, Bedford, MA), and the number of stromal cells was determined by counting in a hemocytometer. Fresh suspensions of stromal cells were plated in six-well, 35-mm culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) at a density of 10^5 cells per dish and kept in an incubator in a humidified atmosphere with 5% CO_2 at 37°C. Media were changed after 48 hours and thereafter at intervals until the cells became confluent, which usually occurred in 5–7 days. Confluent ASC, which assume a fibroblast-like appearance, were then placed in serum-free (SF) Waymouth's media, and the media were changed at least three times over the next 24 hours to remove any remaining serum factors. We obtained CM from MCF-7 and T47-D breast cancer cells (American Tissue Culture Collection, Rockville, MD) as well as SSC 202, SSC 78, and SSC 30 breast cancer cells (kindly provided by Dr. Ali Gadzar, Simmons Cancer Center, University of Texas Southwestern Medical Center, Dallas), and primary cultures of ASC and endometrial stromal cells (kindly provided by Dr. Ann Word, University of Texas Southwestern Medical Center, Dallas). These CM were used to treat ASC in the presence or absence of dexamethasone (DEX, 250 nmol/L) (Sigma) for 24 hours. We obtained CM from breast cancer cells and primary cell cultures after incubation in SF media for 24 hours, to allow accumulation of secreted growth factors from these cells, after a washout period of 24 hours with SF media similar to the protocol for ASC.

T47-D breast cancer cells were grown to confluence in phenol red-free RPMI 1640 medium (Gibco/BRL) and incubated with or without 17β-estradiol (E2, 10^{-7} to 10^{-9} mol/L) or a steroidal estrogen antagonist, ICI 182,780 (Zeneca Pharmaceuticals, Cheshire, UK), for 24 hours, after a 24-hour washout period with SF media. This CM was then used to treat ASC. Alternatively, heat inactivation of CM was performed by placing the CM in a 100°C shaking water bath for 15 minutes and then cooling to room temperature before use.

Aromatase Activity Measurement

Aromatase activity was assayed in intact ASC after the addition of [1β-^{3}H]androstenedione (150 nmol/L) (Dupont, Boston, MA) to the medium. At the end of a 2-hour incubation period, medium was removed, and the incorporation of tritium from [1β-^{3}H]androstenedione

into [^{3}H]water was assayed as described previously in detail.[5] The cells were then scraped from the dishes, homogenized, and assayed for protein using the BCA Protein Assay (Pierce, Rockford, IL). Results are expressed as pmole/mg protein per 2 hours. Each bar represents the mean of results (±standard error of the mean [SEM]) using triplicate sets of dishes.

Isolation of RNA

From ASC maintained in the presence or absence of DEX and in the presence or absence of serum or CM, total RNA was isolated by a single-step method using TRI-SOLV (Biotecx, Houston, TX), following the protocol suggested by the supplier. The RNA concentration was determined by spectrophotometric absorption at 260 nm.

Oligonucleotide Sequences

Synthesized oligonucleotides (oligos) were used as primers and radiolabeled probes.[17] The sequence of the 20-mer 3′ oligo used for initial primer extension was 5′ATTCCCATGGAGTAGCCAGG3′ (AROM III, complementary to coding exon III of the P450arom gene), whereas those of the 5′ oligos used during amplifications were as follows: exon I.3-specific primer, 5′GATAAGGTTCTATCAGACC3′; exon I.4-specific primer, 5′GTAGAACGTCACCAACTGG3′; promoter II-specific primer, 5′GCAACAGGAGCTATAGAT3′; and coding exon II-specific primer, 5′TCTGAGGTCAAGGAACAC3′. The sequences of the different oligos used as [^{32}P]-labeled probes for each of the exon-specific amplification products were as follows: for the exon I.3-specific sequence, 5′GCAGCATTTCTGACCTTGG3′; for the exon I-4-specific sequence, 5′GGTTTGATGGGCTGACCAG3′; for the promoter II-specific sequence, 5′TGTGGAAATCAAAGGGACAGA3′; and for the coding exon III-specific sequence, 5′CAGGCACGATGCTGGTGATG3′.

Reverse Transcription-Polymerase Chain Reaction Amplification (RT-PCR) and Hybridization

Amplification of the 5′ ends of the P450arom transcripts from the various treated and untreated ASC was accomplished with the exon-specific oligo pairs as listed above. Initial primer extension, using 10 μg total RNA, was performed at 37°C for 60 minutes using 100 pmol of the 3′ oligo AROM III, 1 mmol/L final concentration of each dNTP, 5× first strand buffer (Gibco/BRL), 10 mmol/L dithiothreitol, 400 units MMLV-RT (BRL, Gaithersburg, MD), and 40 units of RNAsin (Promega, Madison, WI) in a final volume of 50 μL. Each amplification master mix contained 50 pmol of the 3′ oligo AROM III, 100 pmol of a specific 5′ oligo, 500 μmol/L final concentration of each dNTP, and PCR buffer (Perkin Elmer Cetus, Norwalk, CT), in a final volume of 50 μL (39.5 μL of amplification master mix, 0.5 μL [2.5 units] of AmpliTaq DNA polymerase [Perkin Elmer Cetus], and 10 μL of initial primer extension reaction mix). The mix was amplified for 25 cycles at 93°C for 1 minute, 41°C for 1 minute, and 72°C for 1 minute in a thermocycler (Perkin Elmer Cetus). The amplified products were then treated with DNase-free RNase (Bochringer, Indianapolis, IN) for 30 minutes at 37°C and then size-fractionated (to confirm appropriate size of the amplified products) by electrophoresis on a 1.8% agarose gel and transferred to a blotting nylon membrane (Hybond N+; Amersham, Amersham, UK) by capillary elution in 0.4 mol/L NaOH solution. Southern hybridization with the 5′ termini-specific oligonucleotide probes end-labeled with [^{32}P] was continued overnight at Tm − 5°C for each oligo. Autoradiographs were exposed to blotting membranes for 4–16 hours. Samples containing no RNA were included to preclude product carry-over contamination.

RESULTS

As can be seen in Figure 1, and as documented previously, DEX alone had a minimal effect on aromatase activity of ASC. However, addition of serum created a tenfold increase in aromatase activity, suggesting the presence of a serum-derived aromatase stimulating factor(s). From the results presented here, it is apparent that SF CM from T47-D cells can mimic the effects of serum, as shown by a sevenfold increase in aromatase activity when ASC were incubated in the presence of 100% CM and DEX. Moreover, dilution of the CM with SF medium resulted in a decrease of stimulation in a concentra-

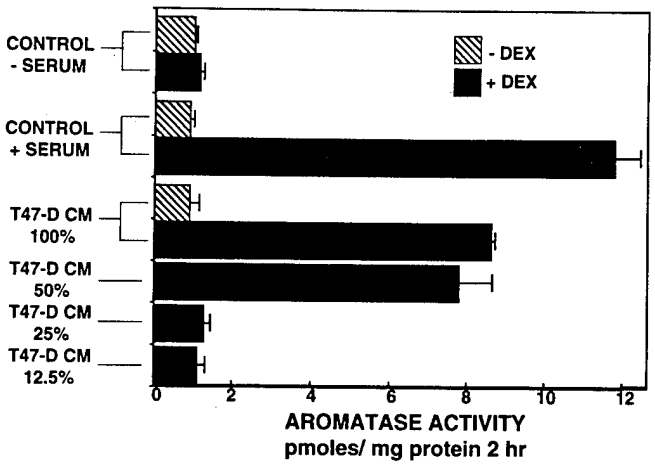

Figure 1. Aromatase activity of human ASC incubated with conditioned medium (CM) from T47-D cells in various dilutions. ASC were maintained in SF medium for 24 hours and then maintained in the presence (*solid bars*) or absence (*shaded bars*) of dexamethasone (DEX) (250 nmol/L) and in the presence or absence of NuSerum (10%) or CM in various dilutions (100% to 12.5%) for a further 24 hours. Aromatase activity was determined after incubation with [1β-^{3}H]androstenedione (150 nmol/L) for 2 hours, as described in Materials and Methods. Results are expressed as pmol [^{3}H]water formed per mg protein per 2 hours, and represent the mean ± SEM of triplicate replicate dishes.

tion-dependent fashion. In addition, aromatase activity of ASC in the presence of CM from T47-D cells but in the absence of DEX was similar to that in the presence of SF media alone (without DEX). Similar effects were seen using CM from MCF-7 cells and SSC 30, SSC 78, and SSC 202 breast cancer cell lines (data not shown).

Figure 2 depicts the effects of CM from T47-D cells treated previously with or without E2 for 24 hours, in concentrations of 10^{-7} to 10^{-9} mol/L, on aromatase activity of ASC in the presence of DEX. As shown, DEX alone caused a modest stimulation of aromatase activity, whereas serum alone was inhibitory. There are consistent observations, but are particularly apparent in this experiment. Pre-treatment of T47-D cells with E2 resulted in a dose-dependent effect, such that CM from E2-treated T47-D cells caused a greater stimulation of aromatase activity of DEX-treated ASC cells than did CM from cells not treated with E2. At 10^{-7} mol/L concentration, E2 led to more than a 1.5-fold stimulation of aromatase activity as compared to no E2 treatment. Furthermore, a steroidal anti-estrogen compound (ICI 182,780) blocked this action of E2 to enhance the aromatase-stimulating activity of CM from T47-D cells. These results are consistent with the hypothesis that estrogen can stimulate T47-D breast cancer cells, which are known to possess estrogen receptors, to secrete factors that can stimulate aromatase activity of ASC.

In addition to CM from several breast cancer cell lines, we also examined the effects of CM from adipose stromal cells per se as well as endometrial stromal cells, both in primary culture. As seen in Figure 3, aromatase activity of ASC, in the presence of DEX, also was markedly stimulated by CM from these nonmalignant cells. This suggests that whatever factor is responsible for this increase in aromatase activity appears to be ubiquitous, or else there are a number of factors that can cause this increase, which may be expressed differentially in different cell types.

Because the stimulatory factor(s) present in both CM and serum most likely represents a peptide growth factor(s), we examined whether it could be destroyed by heating. Figure 4 shows the effects of heat inactivation of CM, which resulted in a complete loss of its ability to stimulate aromatase activity of ASC treated with DEX. Thus, it is likely that the factor(s) in CM responsible for the stimulation of aromatase activity is heat-labile and probably proteinaceous in nature.

We also sought to determine whether the stimulation of aromatase expression by CM in the presence of DEX resulted from the activation of expression of promoter I.4 of the CYP19 gene, similar to the action of DEX in the presence of serum.[17] Total RNA from ASC treated with or without DEX and CM or serum was isolated, and then RT-PCR and Southern hybridization were performed. In Figure 5, the blot probed with the exon I.4-specific probe was exposed for only 4 hours because of its much greater intensity, whereas the blot hybridized with the exon I.3-specific probe was exposed for 16 hours. As can be seen, DEX in the absence of serum stimulated only expression of I.4-specific transcripts. Transcripts presented in untreated cells contained primarily I.3- and PII-specific se-

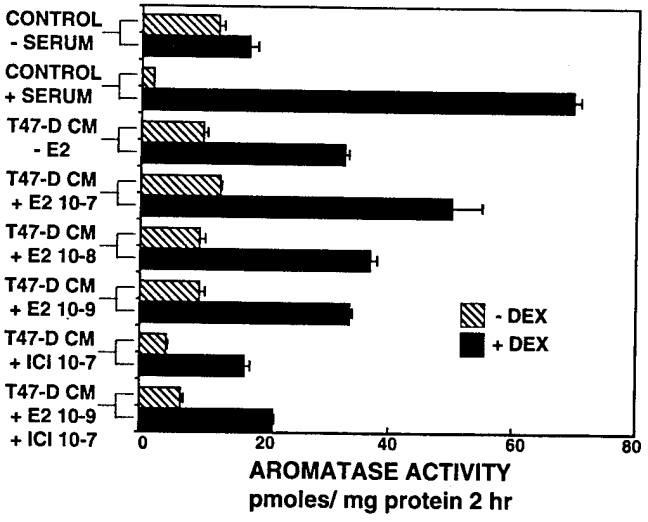

Figure 2. Effect of estrogen on the ability of T47-D conditioned medium (CM) to stimulate aromatase activity of ASC. ASC were maintained in SF medium for 24 hours and then maintained in the presence (*solid bars*) or absence (*shaded bars*) of dexamethasone (DEX) (250 nmol/L) and in the presence or absence of NuSerum (10%) or CM for a further 24 hours. The CM was prepared from T47-D cells treated with estradiol (E2) in various concentrations (0–10^{-7} mol/L) in the presence or absence of ICI 182,780 (10^{-7} mol/L). Aromatase activity of the ASC was determined after incubation with [1β-^{3}H]androstenedione (150 nmol/L) for 2 hours, as described in Materials and Methods. Results are expressed as pmol [^{3}H]water formed per mg protein per 2 hours, and represent the mean ± SEM of triplicate replicate dishes.

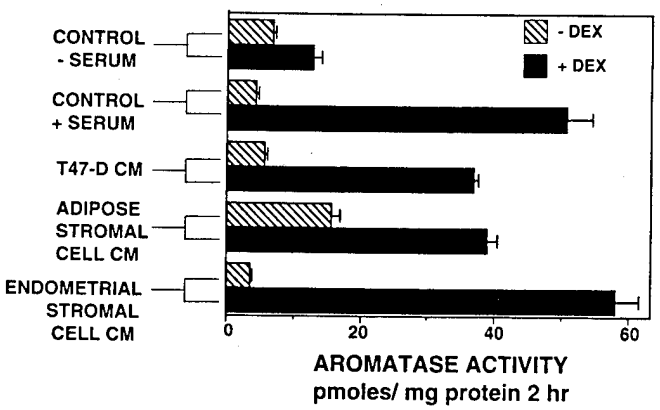

Figure 3. Aromatase activity of ASC incubated with conditioned medium (CM) from various cell types. ASC were maintained in SF medium for 24 hours and then maintained in the presence (*solid bars*) or absence (*shaded bars*) of dexamethasone (DEX) (250 nmol/L) and in the presence or absence of NuSerum (10%) or CM from T47-D cells, ASC, or endometrial stromal cells for a further 24 hours. Aromatase activity was determined after incubation with [1β-^{3}H]androstenedione (150 nmol/L) for 2 hours, as described in Materials and Methods. Results are expressed as pmol [^{3}H]water formed per mg protein per 2 hours, and represent the mean ± SEM of triplicate replicate dishes.

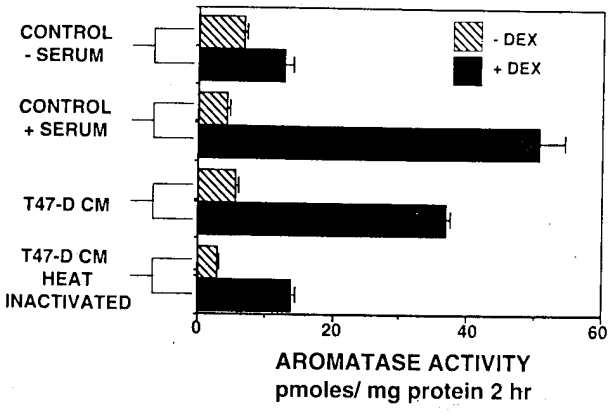

Figure 4. Effect of heat-inactivated conditioned medium (CM) on aromatase activity of ASC. ASC were maintained in SF medium for 24 hours and then maintained in the presence (*solid bars*) or absence (*shaded bars*) of dexamethasone (DEX) (250 nmol/L) and in the presence or absence of NuSerum (10%) or CM from T47-D cells for a further 24 hours. Heat-inactivated CM was prepared as described in Materials and Methods. Aromatase activity was determined after incubation with [1β-^{3}H]androstenedione (150 nmol/L) for 2 hours, as described in Materials and Methods. Results are expressed as pmol [^{3}H]water formed per mg protein per 2 hours, and represent the mean ± SEM of triplicate replicate dishes.

quences (not shown). However, in the presence of DEX and serum, expression of both I.4- and I.3-specific transcripts was noted; by far, the majority of transcripts contained I.4-specific sequences, as expected.[17] In a similar fashion, I.4-specific transcripts predominated in cells treated with CM from T47-D cells.

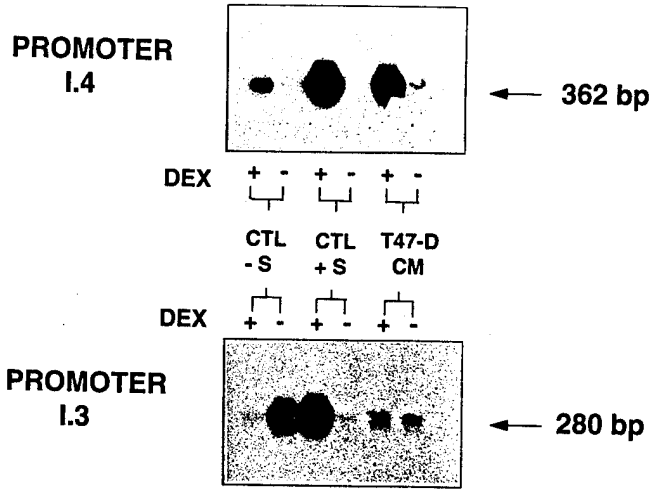

Figure 5. RT-PCR amplification of specific 5′ termini of P450arom transcript in RNA from ASC. ASC were maintained in SF medium for 24 hours and then maintained in the presence or absence of dexamethasone (DEX) (250 nmol/L), NuSerum (10%), or CM from T47-D cells for a further 24 hours. RNA was then isolated and used for RT-PCR of P450arom transcripts with 5′ termini, as described in Materials and Methods. Following electrophoresis and Southern blotting, the filters were subjected to autoradiography. The blot in the upper panel (exon I.4-specific probe) was exposed for 4 hours, whereas that in the lower panel (exon I.3-specific probe) was exposed for 16 hours. CTL = control; S = serum.

DISCUSSION

The results of this study indicate that CM from a variety of cell types and cell lines of human origin are capable of mimicking the effect of serum to stimulate aromatase expression by ASC maintained in the presence of glucocorticoids. Moreover, this activity is likely to be the consequence of a proteinaceous factor or factors secreted by these cells. Cells producing such a factor or factors include MCF-7, T47-D, and SSC-30, -78, and -202 breast cancer cell lines, as well as endometrial stromal cells and ASC themselves, in primary culture. It has been reported previously that another breast cancer cell line, MDA-MB231, also produces a factor(s) with similar properties.[23] Cells producing such a factor(s) are both mesenchymal and epithelial in origin. The stimulatory effect of such factors appears to be due to use of the promoter I.4, whose activity is confined primarily to adipose tissue and fetal liver.[17,24] Thus, the use of RT-PCR indicates that the expression of promoter I-4-specific transcripts is greatly enhanced in the presence of glucocorticoids plus CM, just as it is in the presence of glucocorticoids plus serum.

In previous studies, we and others have shown that aromatase expression in breast adipose tissue proximal to a tumor is greater than at sites distal to the tumor,[13,15] which suggests that cross-talk occurs between a breast tumor and the surrounding adipose tissue in terms of the ability of the latter to synthesize estrogens. These results suggest the possible existence of local paracrine and autocrine loops mediating stromal-epithelial cell interactions, whereby estrogens produced by ASC proximal to a tumor stimulate the tumor cells to produce growth factors. These growth factors in turn stimulate the growth and development of the tumor, but also may act in a paracrine fashion to stimulate the surrounding ASC to produce more estrogens, thus resulting in a positive feedback loop which further supports development of the tumor.[16]

The results of the present experiments support this concept by showing that a number of cell lines, including breast cancer cell lines and ASC themselves, do indeed secrete proteinaceous factors that can stimulate ASC to increase aromatase expression. That several cell types have this ability suggests that a number of factors may stimulate ASC to produce estrogens. Further studies must determine the identities of the factors produced by these various cell types that can stimulate estrogen biosynthesis by ASC via activation of promoter I.4 of the CYP19 gene.

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Aromatase P450 Gene Expression in Human Adipose Tissue

ROLE OF A Jak/STAT PATHWAY IN REGULATION OF THE ADIPOSE-SPECIFIC PROMOTER*

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In the present report we describe a heretofore unrecognized role for a Jak/STAT signaling pathway, namely the stimulation of expression of the aromatase P450 (CYP19) gene, and hence of estrogen biosynthesis, in human adipose tissue. Expression of this gene in adipose tissue as well as in adipose stromal cells maintained in the presence of serum and glucocorticoids is regulated by a distal TATA-less promoter, I.4, which contains a glucocorticoid response element, an Sp1 binding site, and an interferon-γ activation site (GAS) element. The stimulatory action of serum (in the presence of dexamethasone) can be replaced by interleukin (IL)-11, leukemia inhibitory factor, and oncostatin-M, as well as by IL-6, providing the IL-6 soluble receptor is also present. Stimulation of the cells by these factors led to rapid phosphorylation of Jak1, but not Jak2 or Jak3, on tyrosine residues. STAT3 but not STAT1 was also phosphorylated and bound to the GAS element in the I.4 promoter region. When regions of this promoter were fused upstream of the chloramphenicol acetyltransferase reporter gene and transfected into the cells, mutagenesis or deletion of the GAS element led to complete loss of reporter gene expression. Since adipose tissue is the major site of estrogen biosynthesis in men and in postmenopausal women, this pathway involving a Jak/STAT signaling mechanism acting together with glucocorticoids and Sp1 appears to be the principal means whereby estrogen biosynthesis is regulated in the elderly.

Estrogen biosynthesis in humans occurs in a number of tissue sites of expression including the granulosa cells and corpus luteum of the ovary (1, 2), the Leydig cells of the testis (3), the syncytiotrophoblast of the placenta, various sites in the brain including the hypothalamus, amygdala, and hippocampus (4, 5), as well as in adipose tissue (6). The significance of adipose tissue as a source of estrogens was first recognized some 20 years ago by MacDonald, Siiteri, and their colleagues (7–10) who determined the fractional conversion of circulating androstenedione to estrone in male and female human subjects. They found that not only was there a striking increase with obesity, suggesting that most of the extragonadal conversion occurred in adipose tissue, but also that there was an equally striking increase with age for any given body weight.

Estrogen biosynthesis is catalyzed by an enzyme known as aromatase P450 (11–14) (P450arom, the product of the CYP19 gene (15)). CYP19 is a member of the P450 superfamily of genes, which currently contains over 300 members in some 36 gene families (15). Typically, these enzymes catalyze the insertion of oxygen atoms derived from molecular oxygen into organic molecules to form hydroxyl groups. In the case of P450arom, three such attacks by molecular oxygen give rise to loss of the C19 angular methyl group of the steroid substrate as formic acid, with concomitant aromatization of the A-ring to give the phenolic A-ring characteristic of estrogens (16).

A few years ago we and others cloned and characterized the CYP19 gene, which encodes human P450arom (17–19). The coding region spans 9 exons beginning with exon II. Sequencing of rapid amplification of cDNA ends-generated cDNA clones derived from P450arom transcripts present in the various tissue sites of expression revealed that the 5′-termini of these transcripts differ from one another in a tissue-specific fashion upstream of a common site in the 5′-untranslated region (20–22). Using these sequences as probes to screen genomic libraries, it was found that these 5′-termini correspond to untranslated exons that are spliced into the P450arom transcripts in a tissue-specific fashion, due to the use of tissue-specific promoters. Placental transcripts contain at their 5′-ends untranslated exon I.1, which is located at least 40 kilobases upstream from the start of translation in exon II (20, 23). This is because placental expression is driven from a powerful distal placental promoter, I.1, upstream of untranslated exon I.1. On the other hand, transcripts in the ovary contain sequence at their 5′-ends that is immediately upstream of the start of translation. This is because expression of the gene in the ovary utilizes a proximal promoter, promoter II. By contrast, transcripts in adipose tissue contain yet another distal untranslated exon, I.4, which is located in the gene 20 kilobases downstream from exon I.1 (24–26). A number of other untranslated exons have been characterized by ourselves and others (22, 27), including one specific for brain (28). Splicing of these untranslated exons to form the mature transcripts occurs at a common 3′-splice junction, which is upstream of the start of translation. This means that although transcripts in different tissues have different 5′-termini, the protein encoded by these transcripts is always the same, regardless of the tissue site of expression; thus, there is only one human P450arom enzyme encoded by a single copy gene.

Using reverse transcription polymerase chain reaction with an internal standard, we have studied aromatase expression in samples of adipose tissue obtained from women of various ages and have found a marked increase in the specific content of P450arom transcripts in adipose tissue with increasing age,

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thus providing a molecular basis for the previous observation that the fractional conversion of circulating androstenedione to estrone increases with age (29). Furthermore, there are marked regional variations in aromatase expression, with the highest values being found in adipose from buttocks and thighs as compared with abdomen and breast (29, 30).

We also used this reverse transcription polymerase chain reaction technique to examine regional variations in aromatase expression in breast adipose tissue and have found that highest expression occurs in adipose tissue proximal to a tumor, as compared with that distal to a tumor (31, 32). This is in agreement with previous observations regarding the regional distribution of aromatase activity within breast adipose (33, 34) as well as an immunocytochemical study (35). These results suggest there is cross-talk between a breast tumor and the surrounding adipose cells in terms of the ability of the latter to synthesize estrogens and that factors produced by developing breast tumors may set up local gradients of estrogen biosynthesis in the surrounding fat via paracrine mechanisms (36).

We also found that aromatase expression does not occur in adipocytes but rather in the stromal cells that surround the adipocytes, and that may themselves be preadipocytes (37). These stromal cells grow in culture as fibroblasts. Consequently we have employed these cells in primary culture as a model system to study the regulation of estrogen biosynthesis in adipose tissue (38). When serum is present in the culture medium, expression is stimulated by glucocorticoids including dexamethasone (39). Under these conditions P450arom transcripts contain primarily untranslated exon I.4 at their 5′-ends (25). We subsequently have characterized the region of the CYP19 gene upstream of exon I.4 and have found it to contain a TATA-less promoter as well as an upstream glucocorticoid response element and an Sp1 sequence within the untranslated exon, both of which are required for expression of reporter gene constructs in the presence of serum and glucocorticoids (40). Additionally, we found this region to contain an interferon-γ activating sequence (GAS)[1] element. Such sequences are known to bind transcription factors of the signal transducers and activators of transcription (STAT) family (41–43).

In the present study we have observed for the first time that the effect of serum to stimulate aromatase expression in human adipose stromal cells (in the presence of glucocorticoids) can be mimicked by specific factors, namely members of the interleukin-11 (IL-11), oncostatin-M (OSM), IL-6, and leukemia inhibitory factor (LIF) lymphokine family. This stimulation is mediated by a member of the Jak family of tyrosine kinases as well as a STAT transcription factor, which binds to the GAS element within promoter I.4 of the P450arom gene. Thus we have uncovered a regulatory pathway whereby expression of the P450arom gene, via the distal promoter I.4, is stimulated by members of the above cytokine family. Since P450arom transcripts in adipose tissue appear to be derived primarily from expression of promoter I.4 and since adipose tissue is the major site of estrogen biosynthesis in men and in postmenopausal women (44), this pathway composed of a Jak/STAT signaling mechanism acting in conjunction with glucocorticoids and Sp1 appears to be the principal means whereby estrogen biosynthesis is regulated in the elderly.

[1] The abbreviations used are: GAS, interferon-γ activation site; STAT, signal transducers and activators of transcription; IL, interleukin; LIF, leukemia inhibitory factor; OSM, oncostatin-M; RIPA, radioimmune precipitation buffer; PAGE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase; bp, base pair(s); FCS, fetal calf serum.

EXPERIMENTAL PROCEDURES

Materials—Jak1, Jak2, and Jak3 antisera were the generous gift of Dr. James Ihle (St. Jude Children's Research Hospital, Memphis, TN). STAT1 (Cat. number S21120) and ISGR3 (Cat. number G16930) antisera were purchased from Transduction Laboratories (Lexington, KY). The latter antiserum is raised against a mixture of STAT1α and STAT1β. STAT3 antiserum was a generous gift of Dr. Christopher Schindler (Columbia University, New York). IL-11, OSM, LIF, IL-6, and IL-6 soluble receptor were purchased from R & D (Minneapolis, MN). INFα and INFγ were purchased from Sigma. Anti-phosphotyrosine antibody (4 G10) was purchased from UBI (Lake Placid, NY). Herbimycin, H7, and cycloheximide were purchased from Sigma.

Cell Culture and Preparation of Nuclear Extracts—Subcutaneous adipose tissue was obtained from women at the time of reduction abdominoplasty or reduction mammoplasty. Consent forms and protocols were approved by the Institutional Review Board, University of Texas Southwestern Medical Center at Dallas. Adipose stromal cells were prepared as described (38) and maintained in primary culture in Waymouth's enriched medium containing 10% Nu serum (10% v/v) (Collaborative Research Inc.) and allowed to grow to confluence (5–6 days) before treatment. At this time, serum was removed for 24 h, and the cells were treated with 250 nM dexamethasone for 48 h before recombinant human IL-11, OSM, IL-6, and LIF were added at various concentrations and times depending on the different requirements for each experiment. Aromatase activity was determined by the incorporation of tritium into [^{3}H]water from [1β-^{3}H]androstenedione as described previously (38). Cytoplasmic and nuclear extracts were prepared as described by Cooper *et al.* (45) and Dignam *et al.* (46) with some modifications. Cells were cooled on ice, scraped from the plates, washed 3 times with phosphate-buffered saline, and then lysed in modified RIPA (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1% aprotinin, 1 mM phenanthroline, 10 mM pepstein, 0.1% leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 15% glycerol) with protease and phosphatase inhibitors for 30 min on ice. Lysates were precleared by centrifugation for 30 min at 4 °C. For nuclear extracts, after cells were swollen in 2 ml of hypotonic buffer (containing protease and phosphatase inhibitors as in RIPA) they were homogenized with 12 strokes of an all glass Dounce homogenizer (B type pestle). Nuclei were centrifuged, and the pellet was then suspended in 500 μl of chilled buffer (20 mM Hepes, pH 7.7, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% dithiothreitol, and 20% glycerol and protease and phosphatase inhibitors as in RIPA). After centrifugation, the supernatant was stored at −70 °C until used.

Immunoprecipitation and Western Blotting Analysis—Immunoprecipitations were performed essentially as described by Harlow and Lane (47) or following instructions of the manufacturers of the respective antibodies. Lysates were incubated with nonimmune serum and Protein A-Sepharose (50 μl of 50% slurry) for 1 h, followed by centrifugation. Antibodies were incubated with lysates (100 μg) overnight at 4 °C. Immunoprecipitates were isolated with Protein A- or Protein G-coupled agarose or Sepharose (Oncogene Sciences or Sigma) and washed carefully 3 times with the same lysis buffer mentioned above. Proteins in the immunoprecipitates were resolved by 8% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Bio-Rad), and incubated with the appropriate antibody followed by anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase and the enhanced chemiluminescence detection system (Amersham International, U.K.).

DNA Reporter Gene Constructs Containing Deletion Mutations, Site-directed Mutagenesis, and Transient Transfections—Chimeric DNA constructs containing the GAS element in the genomic region flanking the 5′-end of promoter I.4 of the human CYP19 gene were prepared using polymerase chain reaction. These constructs were fused upstream of the chloramphenicol acetyltransferase (CAT) reporter gene. For mutagenesis, an 800-bp fragment containing the GAS element was digested by *Hin*dIII and *Eco*RI and gel-purified to generate the template for mutagenesis. The GAS element was mutated from TTCCTGTGAA to TTCGACTGAA by polymerase chain reaction. The mutated fragment also was fused upstream of the CAT reporter gene. The fidelity of mutagenesis was verified by dideoxy sequencing using a Sequenase DNA kit (U.S. Biochemical Corp.). The transfection was performed by means of calcium phosphate coprecipitation with minor modifications as described (40). Nearly confluent adipose stromal cells in primary culture were transfected with 20 μg of cesium chloride-purified plasmid. Glycerol shock was carried out for 1 min. The cells were allowed to recover overnight in serum-containing media, serum-starved for 24 h, and then treated with 250 nM dexamethasone for 48 h and 10 ng/ml IL-11 overnight. The transfected cells were then harvested and lysed by

means of freeze/thaw 3 times. Total protein was estimated with a kit as recommended by the manufacturer (Pierce). The CAT assay was performed using normalized amounts of cell proteins, which were incubated for 6–8 h at 37 °C. The products of CAT reactions were analyzed by silica gel thin layer chromatography followed by autoradiography.

Electrophoretic Mobility Shift Assay and Southwestern Blotting Analysis—A double-stranded oligonucleotide (5′-GGGTGTTTCCTGTGAAAGTT-3′) was Klenow-labeled using [α-^{32}P]dCTP and then incubated (5000 cpm) with nuclear extracts (5 μg) on ice for 10 min. For the competition assay, the unlabeled oligonucleotide used as competitor was added simultaneously with the labeled fragment at various ratios. The resulting DNA-protein complexes were analyzed by electrophoresis using an 8% polyacrylamide gel with 0.5 × Tris borate-EDTA as running buffer. Southwestern blotting analysis was done following the procedure described by Singh *et al.* (48) with minor modification. Nuclear extracts (60 μg) were fractionated on 8% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. The membrane was processed through a denaturation/renaturation cycle with 6 M guanidine hydrochloride. Then the membrane was hybridized with the Klenow-labeled probe employed in gel shift analysis, followed by washing and autoradiography.

Generally, experiments were performed at least 3 times on separate occasions, and frequently more, to generate consistent results.

RESULTS

IL-11, OSM, and LIF Stimulate Aromatase Activity in Cultured Adipose Stromal Cells in the Presence of Dexamethasone—In previous studies we observed that the stimulatory effect of glucocorticoids on aromatase activity in cultured human adipose stromal cells was manifest only if serum was present in the medium (39). As shown in Fig. 1*A*, a number of lymphokines, namely IL-11, OSM, and LIF can mimic the action of serum to stimulate aromatase activity of adipose stromal cells in the presence of dexamethasone but not in its absence. In the case of IL-11 and OSM, the effect far exceeded that of serum. Following exposure for 24 h, half-maximal stimulation by IL-11 and OSM was achieved at concentrations of ~2–4 ng/ml (~10^{-10} M), although the stimulation by LIF was maximal at 0.5 ng/ml. The time course of stimulation by IL-11 (Fig. 1*B*) was biphasic, with an initial rapid phase lasting 12 h followed by a slower phase extending beyond 48 h of stimulation.

As shown in Fig. 1*C*, IL-6 had no stimulatory activity up to a concentration of 20 ng/ml, either in the presence or absence of dexamethasone. However, a stimulatory action of IL-6 was manifest upon addition of the IL-6 soluble receptor at concentrations ranging from 5 to 20 ng/ml. At concentrations of IL-6 and its receptor of 20 ng/ml each, stimulation was equal to that achieved by adding OSM at a saturating concentration of 10 ng/ml. In other experiments, interferon-α and interferon-γ were found to have no effect to stimulate aromatase activity of these cells (data not shown).

IL-11 Rapidly and Specifically Stimulates Tyrosine Phosphorylation of Jak1 Kinase—To determine whether Jak kinases are involved in the action of IL-11 to activate estrogen biosynthesis in adipose stromal cells, we examined their ability to undergo tyrosine phosphorylation in the presence of IL-11 (Fig. 2). Adipose stromal cells prepared from mammoplasty and abdominoplasty samples and maintained in primary culture were treated with dexamethasone for 48 h with or without IL-11 (10 ng/ml) for 10 min. The cells were lysed, the Jak kinases were immunoprecipitated with the appropriate antibodies, and the immunoprecipitates were resolved on SDS-PAGE. The gels were subsequently blotted to filters and probed with a monoclonal antibody against phosphotyrosine. IL-11 stimulation resulted in the appearance of a band of ~130 kDa, which was immunoprecipitated using antibodies to JAK1 and was undetectable in the absence of IL-11. By contrast, Jak2 kinase was constitutively phosphorylated on tyrosine since the Jak2 band (~130 kDa) was evident prior to the IL-11 treatment, and the level of tyrosine phosphorylation of Jak2 did not change even after IL-11 stimulation. By contrast, tyrosine phosphorylation of Jak3 was not apparent with or without IL-11 treatment. Similar results were obtained using LIF and OSM. Nonimmune serum and an unrelated immune serum failed to precipitate proteins of 130 kDa (data not shown). Additionally, the 130-kDa bands corresponding to Jak1 and Jak2 were not observed when the immunoprecipitations were conducted in the the presence of peptides used to raise these antibodies (data not shown). Probing with a mixture of Jak1, Jak2, and Jak3 antisera showed that no change in the amount of kinase protein occurred within the time frame of the experiment (*lower panel*). It is concluded that in human adipose stromal cells, Jak2 is constitutively phosphorylated on tyrosine residues, and only Jak1 is phosphorylated on tyrosine residues in response to IL-11 treatment under the conditions employed here. A similar situation has been reported to occur in T-lymphocytes stimulated with interferon-α (49).

Characterization of Jak1 Tyrosine Phosphorylation—The kinetics of tyrosine phosphorylation of Jak1 were examined using the same cell lysates as utilized above, and the results are shown in Fig. 3*A*. Phosphorylation was maximal 10 min after IL-11 addition and then subsequently declined. In a study of the concentration dependence of Jak1 phosphorylation (Fig. 3*C*), it was observed that phosphorylation was undetectable employing IL-11 at a concentration of 1 ng/ml. Phosphorylation was detectable when 5 ng/ml IL-11 was used (visible on the original autoradiograph if not on the printed figure) and reached a maximum at a concentration of IL-11 of 10 ng/ml.

Since OSM and LIF also stimulate aromatase activity in adipose stromal cells in primary culture, their action to stimulate tyrosine phosphorylation of Jak1 was also examined. Adipose stromal cells were treated with dexamethasone for 48 h and with FCS (15%), OSM (5 ng/ml), IL-11 (5 ng/ml), and LIF (5 ng/ml) for 10 min. The cells were lysed, the cell lysates were mixed with Jak1 antibody, and the precipitates were resolved by SDS-PAGE and probed with anti-phosphotyrosine monoclonal antibody. The effects of herbimycin A (a tyrosine kinase inhibitor), H7 (a serine/threonine kinase inhibitor), and cycloheximide on Jak1 tyrosine phosphorylation were also examined. As shown in Fig. 4, OSM and LIF could induce tyrosine phosphorylation of Jak1 to about the same extent as IL-11. The tyrosine phosphorylation of Jak1 induced by IL-11 was inhibited by herbimycin A, whereas H7 had no effect. These results further support the concept that the phosphorylation of Jak1 induced by IL-11 is indeed on tyrosine residues. In addition, the rapid phosphorylation of Jak1 was not inhibited by cycloheximide, an inhibitor of protein synthesis, indicative that this is not mediated by the new synthesis of protein factors.

STAT3 Phosphorylation and Binding to the GAS Element in the Upstream Region of Promoter I.4 of the CYP19 Gene—In order to determine whether STAT transcription factors are involved in signal transduction in adipose stromal cells in primary culture in response to IL-11, we examined phosphorylation of STAT1 and STAT3 by immunoprecipitation employing appropriate antisera and subsequent probing with anti-phosphotyrosine monoclonal antibody. Adipose stromal cells prepared from mammoplasty and abdominoplasty samples and maintained in primary culture were treated with dexamethasone for 48 h with or without IL-11 (10 ng/ml) for 10 min. The cells were lysed, and aliquots were mixed with the appropriate antibodies. As can be seen in Fig. 5, a band of 92 kDa was immunoprecipitated by STAT3 antiserum after the cells were treated with IL-11. However, no bands were detectable following immunoprecipitation with antisera raised against STAT1. As

[2] C. Schindler, personal communication.

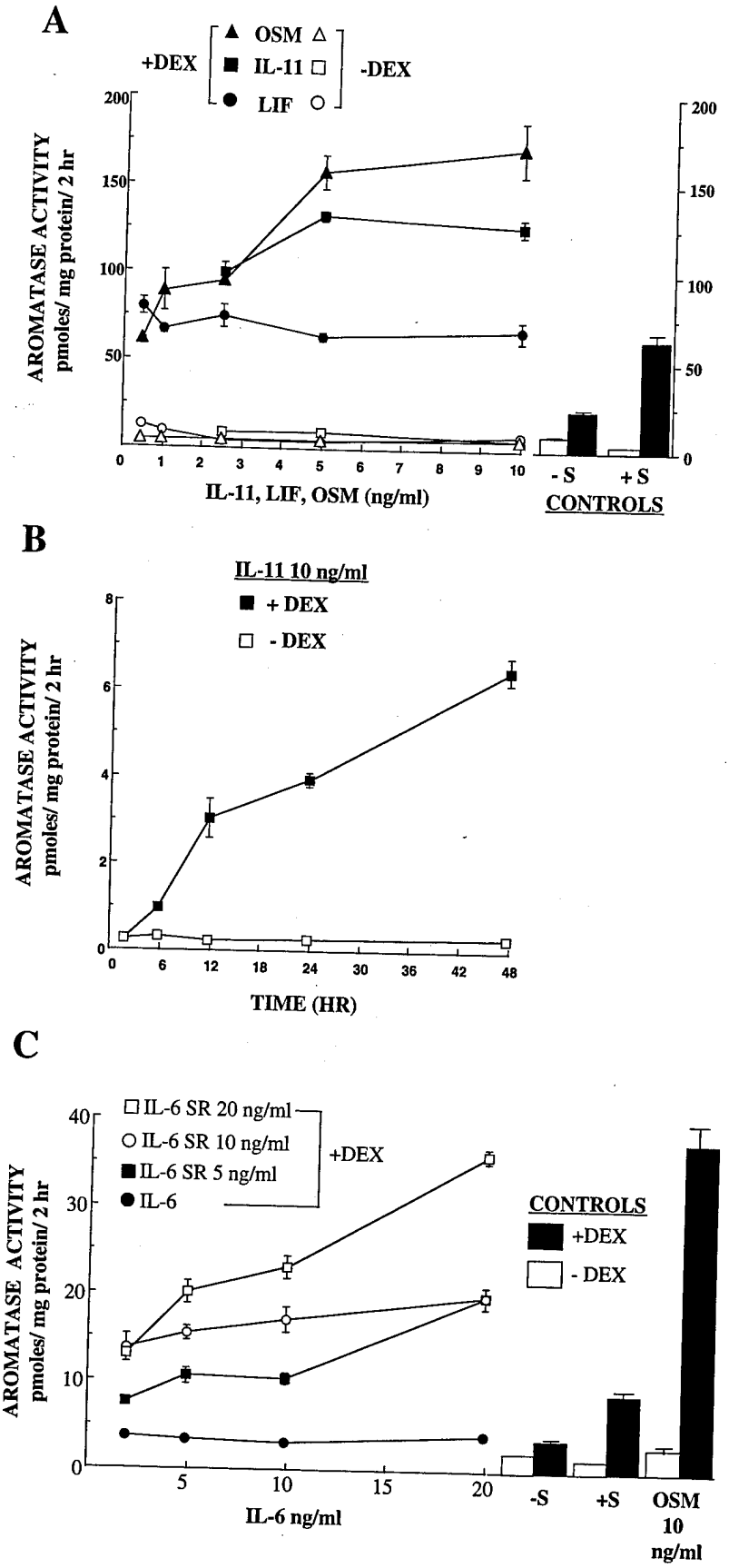

FIG. 1. *A*, concentration dependence of the actions of LIF, IL-11, and OSM to stimulate aromatase activity of adipose stromal cells in the absence (*open symbols*) or presence (*solid symbols*) of dexamethasone (250 nM). Confluent adipose stromal cells in primary culture were maintained for 24 h in the presence or absence of dexamethasone (250 nM) and LIF (*circles*), IL-11 (*squares*), or OSM (*triangles*) in various concentrations. Control dishes incubated in the absence or presence of serum are indicated as ± *S*, with *open bars* indicating the absence and *solid bars* the presence of dexamethasone. *B*, time course of stimulation of aromatase activity of adipose stromal cells by IL-11. Confluent adipose stromal cells in primary culture were maintained for 24 h in the presence (*solid squares*) or absence (*open squares*) of dexamethasone (*DEX*). IL-11 (10 ng/ml) was added to half the dishes, and incubation continued for an additional 48 h. *C*, action of IL-6 and its soluble receptor on aromatase activity of adipose stromal cells. Confluent adipose stromal cells in primary culture were maintained for 24 h in the presence of dexamethasone (250 nM) and in the presence or absence of IL-6 (2–20 ng/ml) and its soluble receptor (*SR*; 5–20 ng/ml). *Solid circles*, no SR; *solid squares*, 5 ng/ml SR; *open circles*, 10 ng/ml SR; *open squares*, 20 ng/ml SR. Control dishes were incubated in the absence or presence of serum (±*S*) or OSM (10 ng/ml) and in the absence (*open bars*) or presence (*solid bars*) of dexamethasone. In each of the experiments shown in *A–C*, aromatase activity was measured at the end of the incubation period as described under "Experimental Procedures." Data are presented as the mean ± S.E. of results from triplicate replicate dishes.

a positive control, we showed by Western blotting that the anti-STAT1 antibody reacted with not only recombinant STAT1 but with STAT1 present in adipose stromal cells treated with dexamethasone in the presence or absence of IL-11 (data not shown). Although the STAT3 antiserum cross-reacts with STAT1α,[2] the use of the commercial STAT1α antiserum ruled out the possibility that the STAT3 antiserum was detecting STAT1.

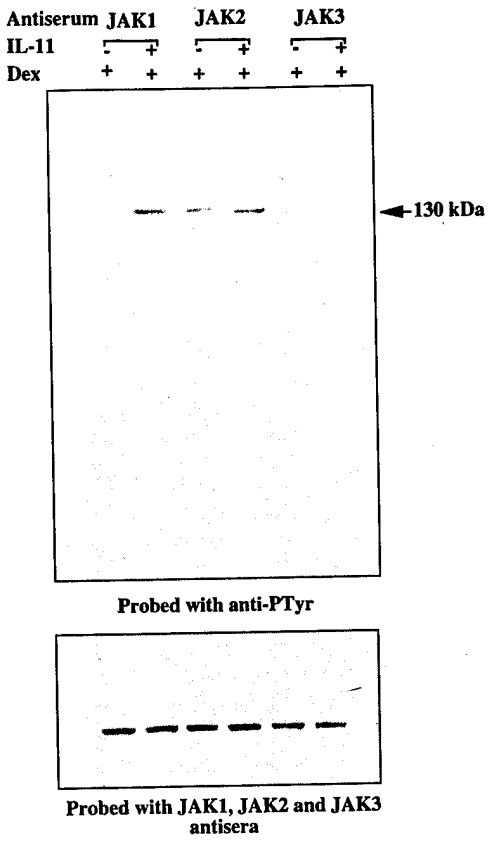

FIG. 2. **Effect of IL-11 on tyrosine phosphorylation of Jak1.** Adipose stromal cells in primary culture were placed in serum-free medium for 24 h. The cells were treated with dexamethasone (*Dex*) for 48 h before addition of IL-11 (10 ng/ml) for 10 min. The cells were collected and washed, and extracts were prepared. Aliquots of extracts (2×10^7 cells) from untreated and treated cells were immunoprecipitated with Jak1, Jak2, or Jak3 antisera as described. The immunoprecipitates were resolved by means of SDS-PAGE and transferred to filters. The filters were probed with the 4G10 anti-phosphotyrosine monoclonal antibody (*top panel*) or with a mixture of Jak1, Jak2, and Jak3 antisera (*lower panel*).

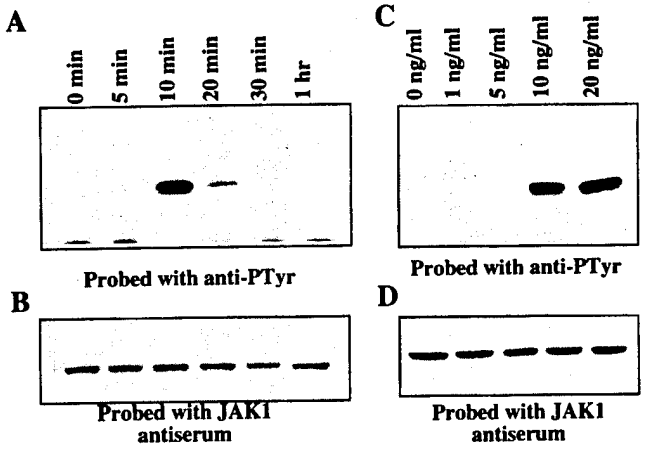

FIG. 3. **Time course and concentration dependence of tyrosine phosphorylation of Jak1.** Adipose stromal cells in primary culture were treated with dexamethasone (250 nM) for 48 h; IL-11 (10 ng/ml) was then added, and incubation continued for 5, 10, 20, 30, and 60 min. Cells were collected at the different time points, and extracts were prepared and then immunoprecipitated with Jak1 antiserum. The immunoprecipitates were fractionated by SDS-PAGE, transferred to filters, and probed with anti-phosphotyrosine monoclonal antibody (*panel A*) or Jak1 antiserum (*panel B*). Cells were treated with dexamethasone for 48 h; IL-11 was added at concentrations of 1, 5, 10, and 20 ng/ml; and incubation was continued for 10 min. Cells were collected, and extracts were prepared. The extracts were precipitated with Jak1 antiserum, and precipitates were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride filter. The transferred proteins were probed with anti-phosphotyrosine monoclonal antibody (*C*) or Jak1 antiserum (*D*). Other details are described under "Experimental Procedures."

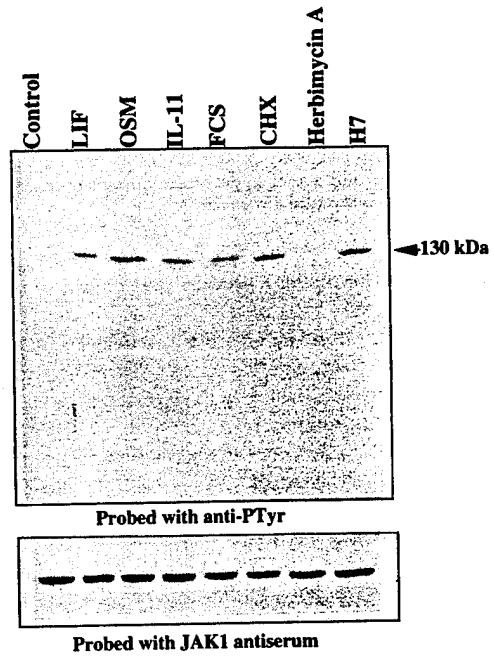

FIG. 4. **Effect of OSM and LIF on tyrosine phosphorylation of Jak1 in adipose stromal cells in primary culture.** Cells were treated with dexamethasone (250 nM) for 48 h; and then OSM (5 ng/ml), LIF (5 ng/ml), IL-11 (10 ng/ml), or FCS (15%) were added, and incubation continued for 10 min. Following treatment with dexamethasone, cells in other dishes were incubated with herbimycin A (5.2 μM) for 14 h, H7 (40 μM) for 30 min, or cycloheximide (*CHX*) (10 μM) for 6 h as indicated. Then IL-11 (10 ng/ml) was added, and incubation continued for a further 10 min. Immunoprecipitation and Western blot analysis were carried out as described under "Experimental Procedures."

To examine the binding activity of nuclear factors to the GAS element of promoter I.4, nuclear extracts were prepared from adipose stromal cells treated with dexamethasone for 48 h and treated with IL-11 for 30 min. Incubation of nuclear proteins from cells treated with IL-11 plus dexamethasone or serum plus dexamethasone with the wild-type promoter I.4 GAS element between −288 and −269 bp as radiolabeled probe (5′-GGGTGTTTCCTGTGAAAGTT-3′) gave rise to a single band (Fig. 6). The band was barely detectable in cells treated with dexamethasone alone. A 100-fold excess of nonradiolabeled consensus GAS sequence resulted in complete competition of the DNA binding (*lane 5*), although use of a mutated sequence (see above) resulted in no displacement (data not shown). In addition, binding of the probe to nuclear protein was displaced when anti-STAT3 serum was added (*lane 7*) but not when anti-STAT1 or control nonimmune sera were employed (data not shown).

Southwestern blot analysis also was performed to further examine the binding of the GAS probe to nuclear proteins prepared from cells treated with IL-11 plus dexamethasone. Nuclear proteins were fractionated by SDS-PAGE and transferred to nitrocellulose membrane. The transferred proteins were subjected to a denaturation/renaturation cycle and hybridized with the radiolabeled probe, followed by autoradiography. As shown in Fig. 7, the radiolabeled GAS probe hybridized to a 92-kDa protein; the intensity of the band was greatly increased when nuclear extracts from cells treated with IL-11 or serum plus dexamethasone were employed, as compared with those treated with dexamethasone alone. The apparent

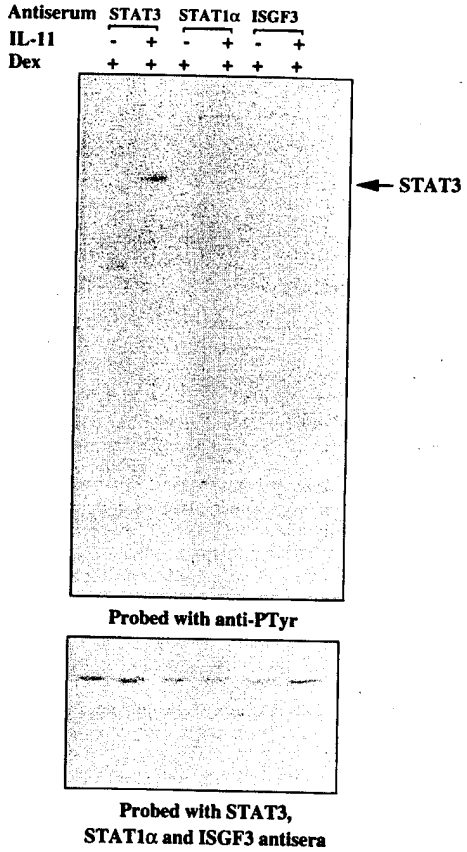

FIG. 5. **Effect of IL-11 on tyrosine phosphorylation of STAT3.** Adipose stromal cells were treated with dexamethasone (*Dex*; 250 nM) for 48 h. IL-11 (10 ng/ml) was then added, and incubation continued for 10 min. Extracts were prepared, and aliquots were immunoprecipitated with appropriate antisera as indicated. Immunoprecipitates were resolved on SDS-PAGE and transferred to a nylon membrane. The transferred proteins were probed with anti-phosphotyrosine monoclonal antibody (*top panel*) or a mixture of appropriate antisera (*lower panel*) as described under "Experimental Procedures."

molecular mass of the nuclear protein that bound to the probe (92 kDa) was similar to that of STAT3 (43). In control experiments, when the mutated GAS sequence was used as probe, no hybridization was detected. Additionally, hybridization to the native probe was also conducted in the presence and absence of a 100-fold excess of native and mutated sequence. Whereas the former resulted in displacement of the radiolabeled probe, the latter did not (data not shown).

The GAS Element Is Essential for Expression of P450arom Fusion Gene Constructs in Adipose Stromal Cells Incubated with IL-11—Genomic constructs containing the wild type GAS, a deletion mutation, and a site-directed mutation fused upstream of the CAT reporter gene were transfected into adipose stromal cells by means of calcium phosphate coprecipitation. Cells were allowed to recover overnight in medium containing 10% serum and then deprived of serum for 24 h followed by dexamethasone treatment for 48 h and IL-11 or serum treatment for 16 h. Cytosolic proteins were prepared, and CAT assays were performed (Fig. 8). CAT reporter activity was undetectable in cells treated with dexamethasone alone, but it was readily apparent in extracts of cells treated with IL-11 or serum plus dexamethasone. CAT reporter gene expression was lost when the GAS element was deleted or mutated to the sequence TTCGACTGAA. These results indicate that the intact GAS element is essential for IL-11- and serum-induced expression driven by promoter I.4.

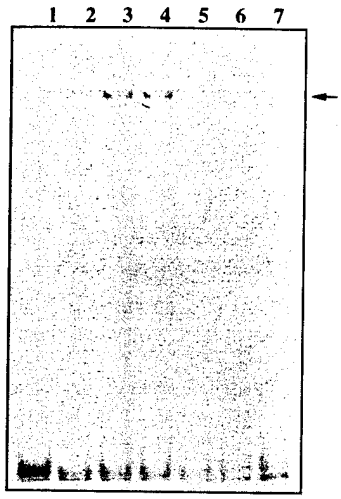

FIG. 6. **Gel mobility shift analysis of proteins binding to the GAS element.** Adipose stromal cells in primary culture were treated with dexamethasone (250 nM) for 48 h and then with IL-11 (10 ng/ml) or serum for 30 min. Nuclear extracts were analyzed by gel shift analysis employing the ^{32}P-labeled −288/−269 bp fragment as probe. Nuclear extracts prepared from cells treated with dexamethasone (250 nM) alone (*lane 2*), IL-11 (10 μg/ml) plus dexamethasone (*lane 3*), or 15% FCS plus dexamethasone (*lane 4*) were incubated with the radiolabeled −288/−269 bp fragment, and the reaction mixtures were subjected to polyacrylamide gel electrophoresis in an 8% gel. For competition, a 100-fold molar excess of the nonradiolabeled −288/−269 bp fragment (*lane 5*) was added to the incubation mixture. To determine whether STAT3 is a component of the protein binding to the GAS, anti-STAT3 serum (2.5 μl) was incubated with the radiolabeled DNA probe in the absence (*lane 6*) or in the presence of nuclear extracts (*lane 7*). *Lane 1*, free probe electrophoresed in the absence of nuclear extract. Other details are described under "Experimental Procedures." The *arrow* indicates the position of the radiolabeled band.

DISCUSSION

The findings of the present study reveal a hitherto unrecognized role for a Jak/STAT signaling pathway, namely the stimulation of expression of the P450arom gene and hence of estrogen biosynthesis in human adipose tissue. The extracellular ligands that initiate this response are members of the IL-11/OSM/LIF family of lymphokines (50, 51). Ligands that have no effect include interferon-α, interferon-γ, and IL-6. However, responsiveness to IL-6 is established upon addition of soluble IL-6 receptor. Members of this lymphokine family employ a receptor system involving two different Jak-associated components, gp130 and LIFRβ, or a related β-component (52). However, the IL-6 receptor complex includes an α component whose cytoplasmic domain is apparently not involved in signaling (52) and which can exist in a soluble form (53). Recently an α-subunit of the IL-11 receptor complex has been cloned (54), although this does not apparently exist in a soluble form. The concentration dependence of the stimulation of aromatase by IL-6, IL-11, LIF, and OSM is indicative of high affinity receptor binding, since half-maximal stimulation was achieved at ligand concentrations of approximately 2 ng/ml (considerably less in the case of LIF), which corresponds to a molar concentration of $\sim 10^{-10}$ M. Of the stimulatory lymphokines, the response to OSM in the presence of dexamethasone was the greatest in terms of aromatase induction and far exceeded the response to serum. Addition of LIF or IL-11 together with OSM resulted in no further increase in stimulation, suggesting that aromatase expression was maximally induced in the presence of OSM and that all of these lymphokines utilized the same signal transduction pathway (data not shown). It should be noted that a variety of other growth factors have no action to stimulate aromatase expression of adipose stromal cells including epidermal growth factor, platelet-derived growth factor, fibroblast

growth factor, growth hormone, prolactin, and IGF-1 (data not shown).

Addition of IL-11 resulted in a rapid phosphorylation of Jak1 kinase in a concentration-dependent fashion, with a maximal effect obtained after 10 min and at a concentration of IL-11 of 10 ng/ml, similar to the concentration of IL-11 required for maximal stimulation of aromatase activity. By contrast, Jak3 kinase was not phosphorylated under these conditions to any significant extent, whereas Jak2 kinase was phosphorylated to an equal extent both in the presence and absence of IL-11. A similar action of interferon-α has been reported in human T-lymphocytes (49). As indicated by blotting with an anti-phosphotyrosine antibody and by inhibition in the presence of herbimycin A, this phosphorylation occurred on tyrosine residues present in the Jak1 kinase. Both gp130 and LIFRβ can associate with and activate at least three members of the Jak family, Jak1, Jak2, and Tyk2, but utilize different combinations of these in different cells (50). From the results presented here, it is apparent that Jak1 is the kinase of choice in human adipose stromal cells. Although there was a rapid phosphorylation of Jak1 on tyrosine residues, Western blot analysis utilizing an antibody against Jak1 indicated that there was no change in the absolute levels of Jak1 throughout this time period of stimulation.

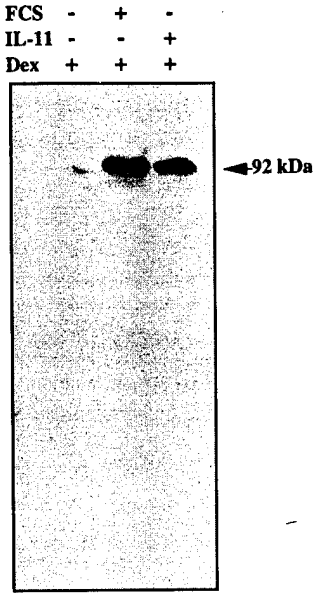

FIG. 7. **Southwestern blot analysis of proteins binding to the GAS element.** Nuclear extracts (60 μg) were separated by SDS-PAGE on an 8% gel, and proteins were transferred to a nitrocellulose membrane. The transferred proteins were subjected to a denaturation/renaturation process and hybridized to the ^{32}P-radiolabeled −288/−269 bp fragment followed by autoradiography. *Lane 1*, nuclear extracts prepared from cells treated with dexamethasone (*Dex*; 250 nM) alone; *lane 2*, nuclear extracts prepared from cells treated with dexamethasone plus IL-11 (10 ng/ml); *lane 3*, nuclear extracts prepared from cells treated with dexamethasone in the presence of serum (15% FCS). Other details are described under "Experimental Procedures."

The action of IL-11 also results in the rapid phosphorylation of STAT3 on tyrosine residues, but this was not the case for STAT1. Recently it has been shown that STAT3 is the substrate of choice for the IL-6/LIF/OSM lymphokine receptor family and that the specificity of STAT phosphorylation is not based upon which Jak kinase is activated (43, 50, 55) but rather is determined by specific tyrosine-based motifs in the receptor components, namely gp130 and LIFRβ, shared by these lymphokines (56). Gel shift analysis, utilizing a double-stranded oligonucleotide corresponding to the wild-type GAS sequence in the promoter I.4 region of the P450arom gene as a probe, indicated binding to a single component. This binding was barely detectable in control cells but was present within 30 min of addition of IL-11 to the cells. This binding was competed by addition of excess nonradiolabeled probe and was also competed upon addition of anti-STAT3 antibody. These results are indicative that STAT3 can interact with the GAS element

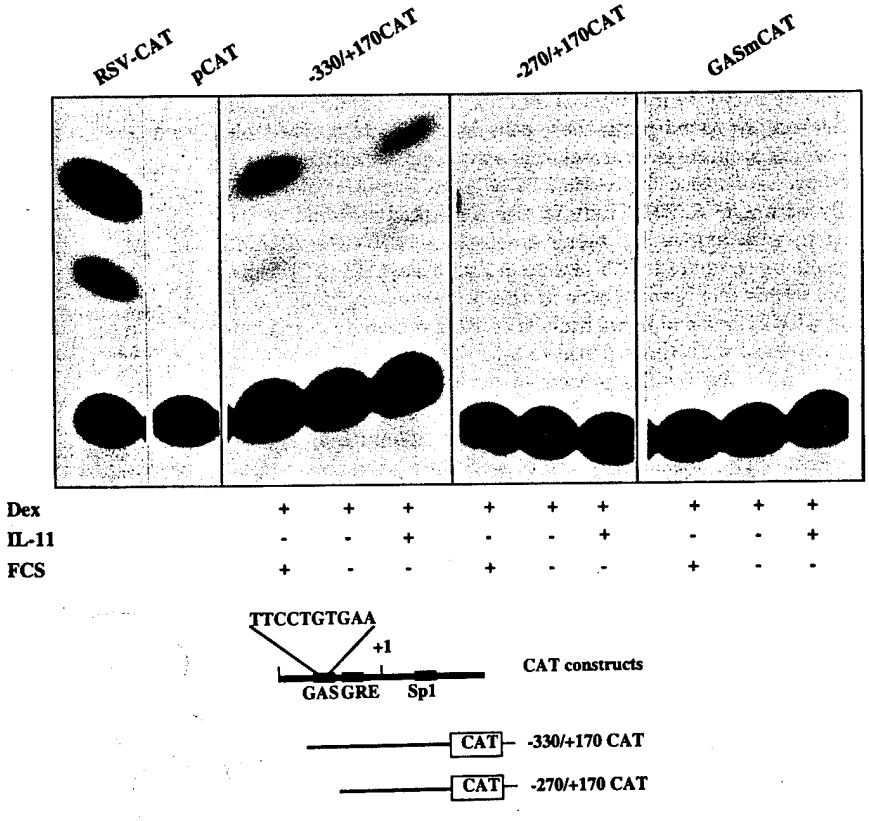

FIG. 8. **Role of the GAS element in transient expression of −330/+170 bp/CAT fusion gene construct.** Fusion gene constructs containing the wild-type −330/+170 bp sequence, the sequence in which the GAS element was deleted (−270/+170 bp), and the mutated GAS sequence (GASmCAT) linked to CAT, were transfected into adipose stromal cells in primary culture. Cells were treated with 250 nM dexamethasone (*Dex*) for 48 h and incubated with or without IL-11 (10 ng/ml) and serum (15% FCS) for 16 h. Cells were then harvested, and lysates were prepared for assay of CAT activity. The products of the CAT reaction were analyzed by TLC followed by autoradiography. RSV-CAT and pCAT are positive and negative vector controls, respectively. Other details are described under "Experimental Procedures."

present in the promoter I.4 region of the P450arom gene upon addition of IL-11 to these cells. This interaction in turn results in activation of expression, as indicated by transfection experiments employing chimeric constructs in which the region −330/+170 bp of the I.4 promoter region was fused upstream of the CAT reporter gene. The results indicate that deletion of the GAS sequence, as well as mutagenesis of this sequence, resulted in complete loss of IL-11- and serum-stimulated expression in the presence of glucocorticoids.

Activation of this pathway of expression by these lymphokines is absolutely dependent on the presence of glucocorticoids. This action of glucocorticoids is mediated by a glucocorticoid response element downstream of the GAS element (40). Additionally, an Sp1-like element present within untranslated exon I.4 also is required, at least for expression of the −330/+170 bp construct (40). These sequences, while present within a 400-bp region of the gene, are not contiguous, and the nature of the interaction among STAT3, the glucocorticoid receptor, and Sp1, to regulate expression of the P450arom gene via the distal promoter I.4 remains to be determined.

Activation of aromatase expression by serum in the presence of glucocorticoids is not confined to cells present in adipose tissue but also has been reported in skin fibroblasts (57) and in hepatocytes derived from fetal liver (58). In each of these cell types the P450arom transcripts contain exon I.4 as their 5′-terminus (27, 59); however, the factors that mimic the action of serum to stimulate aromatase expression in these cell types have as yet to be elucidated. On the other hand, in placenta where the distal promoter I.1 is employed (20) and in ovarian granulosa cells where the proximal promoter II is employed (20, 23), this signaling pathway is not in effect. Thus, aromatase expression in ovarian granulosa cells is driven primarily by cyclic AMP-dependent mechanisms (60).

As indicated previously, adipose tissue is the major site of estrogen biosynthesis in elderly women and men. The fact that this expression is confined to the stromal cells rather than the adipocytes themselves is consistent with the known actions of IL-11 to inhibit the differentiation of 3T3 L1 fibroblasts into adipocytes (61). Since adipose stromal cells are believed to function as preadipocytes and can be converted to adipocytes under appropriate nutritional stimuli, a role of these lymphokines may be to maintain these cells in the preadipocyte state for which aromatase expression is a marker. As indicated previously, aromatase expression in adipose increases dramatically with age (6, 29). There is also a marked regional distribution, with expression being greatest in buttock and thigh regions as compared with abdomen and breast (29, 30). However, within the breast there is also a marked regional variation with expression being highest in sites proximal to a tumor as compared with those distal to a tumor (31, 32).

Based on the results presented here we suggest that aromatase expression in adipose tissue may be under tonic control by circulating glucocorticoids and that regional and age-dependent variations may be the consequence of paracrine and autocrine secretion of lymphokines. Schmidt and Loffler (62), as well as ourselves (64), have shown that conditioned medium from a number of cell types including adipose stromal cells themselves and endometrial stromal cells, as well as breast tumor cells lines, can mimic the actions of serum to stimulate aromatase expression in the presence of glucocorticoids. Thus numerous cell types including breast cancer cells produce factors that are stimulatory of aromatase expression by adipose stromal cells. In preliminary experiments we have shown that a stimulatory factor present in conditioned medium from T47D breast cancer cells can be titrated by an anti-IL-11 antibody (data not shown). Additionally, Reed and colleagues have found that fibroblasts derived from breast tumors secrete IL-6 (34, 63).

Such local paracrine mechanisms could be important in the stimulation of breast cancer growth by estrogens. Commonly, breast tumors produce a desmoplastic reaction whereby there is local proliferation of stromal cells surrounding the tumor, strongly indicative of the production of growth factors by the tumor. These proliferating stromal cells express aromatase, as indicated by immunocytochemistry (35). It is possible then to envision a positive feedback loop whereby adipose stromal cells surrounding a developing tumor produce estrogens, which stimulate the tumor to produce a variety of growth factors and cytokines (64). Some of these act to stimulate the further growth and development of the tumor in a paracrine and autocrine fashion. Additionally, these or other factors act to stimulate proliferation of the surrounding stromal cells and expression of aromatase within these cells. Thus a positive feedback loop is established by paracrine and autocrine mechanisms, which leads to the continuing growth and development of the tumor (32, 36). Further insight into the paracrine mechanisms involved in regulation of estrogen biosynthesis in human adipose tissue will await the characterization of the particular cytokines that are being produced as well as their levels of expression, both of which may vary in a region- and age-dependent fashion.

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Quantitative detection of alternatively spliced transcripts of the aromatase cytochrome P450 (*CYP19*) gene in aromatase- expressing human cells by competitive RT-PCR

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ABSTRACT

C19 steroids are converted to estrogens in a number of tissues by a specific form of cytochrome P450, namely aromatase P450 (P450arom; the product of the *CYP19* gene). The human *CYP19* gene comprises nine coding exons, II-X. The tissue-specific expression is determined by the use of tissue-specific promoters, which give rise to P450arom transcripts with unique 5′-untranslated sequences. The majority of the transcripts present in the ovary contain promoter II-specific sequences, while transcripts in the placenta contain exon I.1. Transcripts in adipose tissue posses exon I.3 and exon I.4. Also, the distribution of alternative transcripts in adipose stromal cells depends on the culture conditions. Therefore, a competitive RT-PCR method was designed to quantitatively detect alternatively spliced transcripts present in various tissues and cells maintained in different culture conditions. Specific synthetic transcripts with different 5′-termini (exon I.3, exon I.4 and promoter II-specific sequences) and the coding region were used as internal standards. This competitive RT-PCR method was used to quantitatively detect three 5′-termini, i.e. promoter II-specific sequence, exon I.3 and exon I.4, in transcripts in human adipose stromal cells and ovarian granulosa cells in primary culture. The quantity of total P450arom transcripts was judged by amplifying the coding-region. We were also able to quantify rare transcripts which could not be detected previously by northern analysis.

Keywords: alternatively spliced transcripts, competitive RT-PCR, human, aromatase cytochrome P450 (*CYP19*) gene

INTRODUCTION

The biosynthesis of estrogens from androgens is catalyzed by an enzyme termed aromatase cytochrome P450 (P450arom; the product of the *CYP19* gene) which is localized in the endoplasmic reticulum of cells in which it is expressed [1-5]. This enzyme is responsible for binding the C_{19} steroid substrate and catalyzing the series of reactions leading to the formation of the phenolic A ring characteristic of estrogens [6-10].

In most vertebrate species examined, aromatase expression occurs in the gonads and in the brain. In the case of humans, the aromatization reaction occurs in a number of cells and tissues including ovarian granulosa cells [11], testicular Sertoli [12] and Leydig cells [13,14], placenta [2,3], adipose tissue of both males and females [15], and various sites of the brain including the hypothalamus [16], amygdala and hippocampus [17]. However, the principal sites of estrogen biosynthesis in the human female are the ovarian granulosa cells in premenopausal women, the placenta in pregnant women, and adipose tissue in postmenopausal women. The physiological significance of estrogen biosynthesis in human placenta and adipose tissue is unclear at this time; however, the latter has been implicated in a number of pathophysiologic conditions. Estrogen biosynthesis by adipose tissue not only increases as a function of body weight but also as a function of age [18,19]. Further, there appears to be a relationship between estrogen biosynthesis in adipose and several disease states such as postmenopausal endometrial and breast cancer [20,21].

The human *CYP19* gene encoding aromatase cytochrome P450 has been cloned and spans more than 75 kilobases (kb) in size; however, the coding region is about 35 kb and contains nine exons (II-X). The heme-binding region is located on the last coding exon (X), and the translation initiation site is located in exon II. Upstream of exon II are located a number of untranslated exons which are spliced into the 5′-ends of transcripts in a tissue-specific fashion [22]. For this reason, specific 5′-termini are found in the transcripts encoding P450arom in different tissues. All of the sequences are spliced onto exon II at the same 3′-splice junction, upstream of the start of translation, and thus the coding region and the protein are the same regardless of the tissue-site of expression. The majority of the transcripts present in the ovary contain sequences specific for promoter II, whereas transcripts in the placenta contain sequences specific for exon I.1. Adipose tissue

expresses two species of transcripts, containing exon I.3- and I.4-specific sequences [23]. Also, the distribution of alternative transcripts in adipose stromal cells [23] depends on their culture conditions. Thus, the cytochrome P450arom gene encodes a number of alternatively spliced transcripts in various tissues regulated by tissue-specific promoters. In order to comprehend the regulation of the *CYP19* gene in any given tissue, it is very important to have a quantitative estimation of the various transcripts containing the different 5′-termini present in that particular tissue. The RACE (Rapid Amplification of cDNA ends) procedure was utilized previously to amplify alternative 5′-termini sequences from P450arom transcripts [23]. However this method may not amplify different transcripts with equal efficiency, and thus may not yield a true quantitative distribution of the various 5′-termini in *CYP19* gene transcripts in a particular cell or tissue. Northern analysis can detect only abundantly expressed transcripts. Therefore, considering the complexity of the expression of alternatively spliced transcripts of the *CYP19* gene, it became essential to design a sensitive method to detect the 5′-termini of various transcripts.

Here, we describe a competitive RT-PCR method to quantify three major 5′-termini of *CYP19* gene transcripts, i.e. promoter II-specific, exon I.3- and exon I.4-specific. Using this method we were able to quantitatively detect multiple transcripts present in primary cultures of human adipose stromal cells and ovarian granulosa cells grown in different culture conditions. Also, for the first time, we report analysis of the alternative transcripts present in cultured adipose stromal cells treated with dexamethasone and the cytokine IL-11. Most importantly, this method is applicable to quantitative analysis of alternatively spliced transcripts of other genes in which this form of regulation of expression is utilized.

MATERIALS AND METHODS

Internal Standards

Figure 1 schematically depicts the principle and steps involved in generating internal standards which were used as competitors for RT-PCR of human P450arom transcripts. To prepare internal standards, three PCR products (AP-PII, AP-I.3 and AP-I.4) were amplified from partial cDNA clones (RACE clones[23]) specific for promoter II-specific sequence, exon I.3 and exon I.4, respectively. RT-1, RT-2, RT-3 (sense oligos specific for each untranslated exon) and RT-4 (antisense oligo for exon II) were used as primers, and their sequences are given in Table 1. Simultaneously, one PCR product (AP-Cod) was amplified from a cytochrome P450arom cDNA clone [24] using the sense primer (RT-5) from the non-coding region of exon II and the antisense primer (RT-6) from exon IV. PCRs were set up as described in the detailed protocol of RT-PCR (see following paragraph), except [α-^{32}P] dCTP was not included, and were performed on a Gene Amp PCR system 9600 (Perkin-Elmer). Reaction conditions were as follows: denaturing temperature 93^{0}C for 1 min, annealing temperature 41^{0}C for 1 min, extension temperature 72^{0}C for 1 min, total cycles 30. By combining one of each of the AP-PII, AP-I.3, AP-I.4 (PCR products) with AP-Cod (PCR product), PCR was used to amplify three different sequences containing alternate 5′-termini, *i.e.* promoter II-specific sequence, exon I.3 or exon I.4. The antisense primer (RT-13, Table 2) contains a 27 bp random AT rich region (loop[25]) and was designed to include a sequence specific to exon III on either side of the 27 bp loop region (Fig. 1). Sense primers (RT-9, RT-10, RT-11) were 5′-termini specific. Primers used in these reactions are listed in Table 2. A similar reaction was set up for amplifying part of the coding region (Exon II-Exon III) using primers RT-12 and RT-13 (Table 2) and a P450arom cDNA clone [24] as a template. CUA tails in the sense primers (5′-end primers, RT-9, RT-10, RT-11, RT-12) and a CAU tail in the antisense primer (3′ end primer, RT-13) were incorporated according to the CloneAmp™ System instructions (Gibco-BRL). The PCR products were then cloned into the pAMP-1 vector. These constructs were transcribed using SP6 polymerase according to the Promega Kit (Madison, WI). Thus four internal standard transcripts were synthesized, each with the 27 bp random AT rich loop. Three of them are specific for the three major 5′-termini of the

CYP19 gene, i.e. exon I.3, I.4 and promoter II-specific sequences and one transcript specific for the coding region between exon II and III. A known copy number of these transcripts was added to each RT-PCR reaction containing target RNA from the various tissues studied.

RT-PCR

RNA samples were treated with DNase I (Gibco-BRL) at room temp for 15 min to remove DNA contamination from the RNA samples. DNase I was inactivated at 90°C for 5 min. Then RNA was denatured at 65°C for 2 min and annealed to random hexamers (Gibco-BRL) by allowing the samples to cool slowly to 37^0C. To synthesize the complementary DNA (cDNA), the annealed RNA was reverse-transcribed using Superscript II (Gibco-BRL) at 42°C for one h in the presence of 166 μM of each dNTP, 50 mM TrisHCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 5 units of RNasin (Promega). cDNA was amplified using the polymerase chain reaction in the presence of 200 μM of each dNTP, 4mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.001% (w/v) gelatin, 10 pmole of each primer, 0.3 μCi of [α-^{32}P]dCTP (Amersham) and 2 units of Taq Polymerase (Perkin-Elmer) in a total volume of 10 μl. RT-1, RT-2, RT-3 and RT-7 primers (Table 1), specific for exon I.4, exon I.3, promoter II-specific sequence and coding exon II respectively, were used as 5′-end sense primers. RT-8 primer (Table 1) specific for exon III was used as 3′-end antisense primer. PCR conditions were: denaturing at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 30 s, for 25 cycles and were performed on a Gene Amp PCR system 9600 (Perkin-Elmer). One fourth of the PCR products was analyzed on 4% non-denaturing polyacrylamide gels. Labelled molecular weight marker (pBR322-Msp1-digested, New England Biolabs) was loaded simultaneously on the gel to assess the sizes of the amplified products. The molecular weight marker was end-labelled using 10 units of T4 polynucleotide kinase (Gibco-BRL) in the presence of 70 mM TrisHCl (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl, 1mM of 2-mercaptoethanol and 30 μCi of [γ-^{32}P] ATP (Amersham). Gels were either autoradiographed with Kodak X-Omat film (Eastman Kodak, Rochester, NY) or scanned

on a PhosphorImager (Molecular Dynamics, CA) and quantitatively analyzed using ImageQuant software. Expected sizes of PCR products are listed in Table 3.

To check the integrity of cDNA, the 'housekeeping' gene, glyceraldehyde -3-phosphate dehydrogenase (GAPDH) was chosen as an endogenous marker. Primers were designed from the sequence deposited in Genbank (Table 1). cDNAs were amplified in the presence of 2 pmoles of each primer, 200 μM of each dNTP ,10 mM TrisHCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% (w/v) gelatin, 0.3 μCi of [α-^{32}P] dCTP (Amersham) and 2 units of Taq polymerase (Perkin-Elmer) at 94°C for 1 min and 72°C for 1 min for 22 cycles.

RNA Isolation

Total RNA was isolated from adipose stromal cells according to the described method with minor modifications [26]. Dishes of frozen confluent adipose stromal cells were scraped in guanidinium thiocyanate. Total RNA from granulosa cells was extracted as described [27]. Total RNA pellets were resuspended in diethylpyrocarbonate (DEPC)-treated water and then precipitated with the addition of ethanol and potassium acetate [26].

Cell Culture

Subcutaneous adipose tissue was obtained from women at the time of reduction mammoplasty. Follicles were obtained from women undergoing hysterectomy or bilateral oophorectomy for benign gynecological disease. Written consent was given preoperatively for the use of these tissues using a consent form. Consent forms and protocols were approved by the Institutional Review Board, University of Texas Southwestern Medical Center at Dallas. Adipose stromal cells were isolated and maintained as primary cultures in Waymouth's enriched medium containing Nu Serum (15%, v/v) (Collaborative Research Inc.) as described previously [28]. Upon reaching confluence, the cells were placed in serum-free or FCS-containing (15%, v/v) Waymouth's enriched medium for 24 h. The cells were then treated with dexamethasone (250 nM) in medium containing 15% FCS for 48 h to maximally induce P450arom mRNA levels. For treatment with dexamethasone and IL-11, IL-11 (10 ng/ml) was added to the culture

medium after dexamethasone treatment and cells were incubated for 24 h. Media were removed, and the cells were frozen at -70^{0}C until used for RNA isolation. Human granulosa-lutein cells were obtained and cultured as previously described [29]. Cultured granulosa cells were treated with 10 μM forskolin for 48 h.

RESULTS

Herein we describe a sensitive quantitative RT-PCR method to detect three major alternatively spliced transcripts of the *CYP19* gene in human adipose stromal cells and granulosa cells. By using this method, we were also able to amplify the 5′-termini of transcripts which were present in very low copy number in tissues in the presence of other abundantly expressed transcripts. Before discussing these results in detail, we will describe the standardization and validation of our RT-PCR method.

A number of experiments were performed to select one set of PCR conditions and reagent concentrations (oligonucleotides, dNTPs and Mg^{2+}) which efficiently amplified all three 5′-ends of alternatively spliced transcripts (data not shown). Comparative efficiency of PCR amplifications for the 5′-termini, i.e. promoter II-specific sequence, exon I.3, exon I.4 and the coding region of the P450arom gene, were determined experimentally. For these experiments, we used only the internal standard transcripts (synthetic transcripts), because the only difference between synthetic transcripts and target sequences was the 27 bp random AT rich sequence inserted into the synthetic transcripts. Our primers do not include that sequence and are similar for target and synthetic transcripts. Therefore, amplification of the synthetic transcripts will reflect the efficiency of amplification of the corresponding unknown target transcripts. Experiments were designed to address each of the following issues:

1) The quantitative nature of PCR has previously been questioned [30,31]. This conclusion was largely based on techniques which involved a high number of PCR cycles. To introduce more sensitive methods for detecting PCR products, submaximal numbers of cycles have been used which lie in the linear range of amplification. Amplification of the 5′-terminus of each transcript was tested after 15, 20, 25, 30 and 35 cycles by using the appropriate specific primers (Fig. 2). Twenty five cycles were found to be the most appropriate because amplification under these conditions is within the linear range and is also submaximal. All experiments described hereafter employed amplification for 25 cycles.

2) The amplification efficiency of transcripts containing three alternate 5′-termini (e.g. exon I.3, I.4 and promoter II-specific) and the coding region may not be equal. To

answer this question, equal quantities of all four synthetic transcripts were reverse transcribed according to the protocol described in Materials and Methods and amplified by PCR using their corresponding primers (Table 1). Results are shown in Figure 3. As expected, amplified bands were of expected size and of almost the same intensity (Fig. 3A). Equal quantities of all transcripts used were confirmed by amplification of the coding region (exon II-exon III) specific sequence which was common to all transcripts (Fig. 3B).

3) Specific amplification of each 5′-terminus from the mixture of P450arom transcripts containing alternate 5′-termini may not be possible. To ensure that the products generated in the RT-PCR method were specific for the desired 5′-termini, we set up four PCRs using the cDNA of one synthetic transcript (internal standard), e.g. exon I.3-specific transcript, and four sets of primers for exon I.3, I.4, promoter II-specific sequence and the coding region in each reaction (Fig. 4). Similar reactions were also set up for the cDNAs of the other three synthetic transcripts, i.e. for exon I.4, promoter II-specific and coding region. As was expected, no cross reaction (amplification) was detected, not even weak amplification after very long exposure. Thus, each synthetic transcript specific for one 5′-terminus was only amplified by primers specific for that sequence and for the coding region (Fig. 4).

4) The amplification efficiencies of each transcript in the presence of the other alternatively spliced transcripts may not be equal. One picogram of each of the three synthetic transcripts, exon I.3, exon I.4 and promoter II-specific, were mixed together. To approximate *in vivo* conditions, 50 ng of sheep kidney (a tissue lacking *CYP19* gene expression) RNA was added. The mixture was reverse transcribed, divided into four tubes and amplified by using the four sets of primers (Fig.5). Amplification efficiencies of exon I.3, exon I.4 and promoter II-specific sequences were almost similar. As was expected, amplification of the coding region was approximately three times higher in comparison to the others because the coding region is common in all synthetic transcripts.

5) The most important question was: when varying quantities of alternatively spliced transcripts are present, will the amplification efficiency of the 5′-terminus of any one transcript be affected. To answer this question, we designed nine experiments (Table 4). Varying quantities of each synthetic transcript were mixed with a constant amount of

the other two transcripts resulting in nine different combinations. Sheep kidney RNA (50 ng) was added in each experiment to approximate an *in vivo* situation. Results are shown only for two experiments using varying concentrations of promoter II-specific transcripts (Fig. 6). Similar experiments were performed with varying concentration of exon I.3 and exon I.4-specific transcripts (data not shown). We concluded that the amplification efficiency of the 5′-terminus of one transcript as a function of its concentration is not affected by the presence of different concentrations of other alternate transcripts (Fig. 6).

For standardization purposes, we used synthetic transcripts (internal standard) alone because they reflect the amplification efficiency of target sequences. When these synthetic transcripts were mixed with unknown target RNA, they should compete so that by comparing amplification of known quantities of internal standard, quantities of unknown transcripts can be calculated. Our goal was to quantitatively detect the 5′-termini of the several alternatively spliced transcripts from cells grown under different culture conditions as well as from tissues. Thus, we mixed three different quantities of internal standard transcript (coding-region specific) with the same quantity of RNA from adipose stromal cells grown in the absence of serum. As was expected, two amplified fragments were observed (Fig. 7). Internal standard cDNA amplification (221bp) was competing with target cDNA amplification (194 bp). Identical experiments were performed using internal standard transcripts specific for exon I.4, exon I.3 and promoter II-specific sequences, and similar patterns were obtained (results not shown).

Results of the experiments using cultured cells are shown in Figure 8 and Table 5. Quantification of each transcript in the cells was performed using 2-3 different concentrations of internal standard. The concentration at which amplified products of target and synthetic transcripts were equal was considered the concentration of target transcripts (corrected for size difference because molar ratio is equal). Because of the complexity of the gel, the data showing amplified products from target and internal standard transcripts for each 5′-terminus is not shown but results are presented in Table 5.

In human adipose stromal cells cultured in the absence of serum, the amplification products of promoter II and exon I.3 (truncate)-specific sequences appeared to be similar in abundance. There was no amplification of exon I.4-specific sequences (Fig. 8A). In

adipose stromal cells treated with dexamethasone in the presence of serum, we observed good amplification of exon I.4 and I.3 (truncate)-specific sequences. There was very low expression of promoter II-specific transcripts (Fig. 8B, Table 5). Transcripts containing exon I.4-specific sequences and exon I.3 (truncate)-specific sequences were almost equal in abundance (Table 5). Total P450arom transcript expression (detected by amplifying the coding region) was about four times higher than in adipose stromal cells grown in the absence of serum (Table 5). When human adipose stromal cells were treated with dexamethasone and the cytokine IL-11, total P450arom transcripts were present in a high copy number, that was similar to the abundance of total P450arom transcripts present in cells treated with dexamethasone plus serum (Table 5). All three 5′-termini were amplified (Table 5). Although transcripts containing exon I.4-specific sequences were the most abundant, promoter II- and exon I.3 (truncate)-specific transcripts were also present in higher copy number in comparison to cells treated with dexamethasone alone.

In primary cultures of human ovarian granulosa cells treated with forskolin for 48 h, promoter II-specific sequences were those mainly amplified (Fig. 8C, Table 5). Transcripts containing exon I.3 (truncate)-specific sequences were in very low copy number (Table 5). Transcripts containing exon I.4 were undetectable.

DISCUSSION

Tissue-specific expression of the human *CYP19* gene appears to be regulated by tissue-specific promoters in the ovary, placenta and adipose tissue [22]. This conclusion is based on the presence of specific 5′-termini present in transcripts encoding P450arom in each of these tissues. Transcripts specific for proximal promoter II-specific sequence are found in the ovary, whereas transcripts specific for the distal promoter I.1 are found uniquely in placenta. Adipose tissue contains mainly two species of transcripts with exon I.3 and I.4-specific sequences. On the other hand, when adipose stromal cells are maintained under different culture conditions [23], different 5′-termini are found. Therefore, it became very important to design a very sensitive, rapid and quantitative method to detect multiple alternatively spliced transcripts present in various human tissues and cells grown in different culture conditions. Thus, we designed a quantitative RT-PCR method in which we used four different internal standards (synthetic transcripts) specific for transcripts containing each 5′-terminus, i.e. promoter II-specific sequence, exon I.3-, exon I.4-containing transcripts, as well as the coding region. Use of internal standards in quantitative RT-PCR has become common these days, but our method to synthesize internal standards by inserting a 27 bp random sequence in combination with direct cloning using the CloneAmp™ System (Gibco-BRL), is very rapid, especially since it was necessary to prepare multiple internal standards. Using trace amounts of [α-^{32}P]-dCTP in the PCR reaction, it was possible to resolve the reactions on a 0.3 mm nondenaturing polyacrylamide gel which was dried and exposed to either X-ray film or phosphorimager for quantification. This technique yielded quick and accurate results, and additionally, it was possible to distinguish amplified bands differing by only 5-6 bp.

In addition to standardization of these basic techniques, the aim of our research was to amplify multiple 5′-termini of alternate transcripts using the same reaction conditions, since employing separate reaction conditions for each specific 5′-terminus would have proved difficult for two reasons: 1) Setting up separate PCRs could have resulted in pipetting errors which are more likely to occur for smaller volumes, while this can be minimized by making master mixes. 2) Setting up separate PCRs is time consuming, especially when the sample number is very high. Because we have to amplify

the 5′-termini of the transcripts of many samples, To simplify the procedure, we decided to normalize one set of PCR conditions for amplification of three 5′-termini, *i.e.* promoter II-specific sequences, exon I.3, exon I.4 and the coding region. To check the validity of these reaction conditions, we performed a number of experiments using synthetic transcripts (internal standards). The same primer pairs were able to amplify sequences from both target RNA and synthetic cRNA. The only difference was that products amplified from synthetic RNA were 27 bp larger in size.

Further, when using the same conditions for four sets of primers (specific for each 5′-terminus) in which the antisense oligo was the same, there was a high possibility of cross reaction. However, no cross amplification was observed (Fig. 4). Efficiencies of amplifications of the 5′-termini were also checked by mixing equal quantities (1 pg) of synthetic transcripts (Fig. 5). This was very important information, since we were trying to amplify one 5′-terminus in one tube from the mixture of several 5′-termini. We found equal efficiencies of all three 5′-termini amplified. Further, when we amplified coding region-specific sequences, we observed three times more amplified product as of untranslated exons (Fig. 5). This was the anticipated result, since the coding region amplified product should be the sum of all three transcripts used, as it is common to all synthetic transcripts. The concentration of these transcripts in 1 μg of cultured cells was also in a similar range (Table 5), thus we could conclude that amplification efficiencies of all three untranslated exons were equal in our experiments using human adipose stromal cells and granulosa cells.

Similarly, we performed experiments using various combinations of the three synthetic transcripts containing promoter II-specific sequence, exon I.3 and exon I.4 (Table 4, Fig. 6). We observed that the efficiency of amplification of one 5′-terminus was not changed in the presence of varying concentrations of the other two 5′-termini. This was a very important point when trying to amplify and quantify multiple 5′-termini from alternatively spliced transcripts present in a tissue. Further, competition experiments were also performed and satisfactory results were obtained (Fig.7) showing competition between target cDNA and cDNA of internal standard transcripts. Thus, we were able to

calculate the amount of unknown target transcript by comparing amplified products with the amplified products of known quantities of internal standard transcripts added in the reaction.

After satisfactory standardization of the PCR conditions, we amplified different 5′-termini of transcripts present in human adipose stromal cells and ovarian granulosa cells in primary culture. Previously we observed that the distribution of the various 5′-termini in adipose stromal cells appeared to be a function of the culture conditions under which the cells were maintained [23]. By northern analysis, we could detect 5′-termini of only abundantly expressed transcripts. We also amplified 5′-terminal sequences by means of the RACE method [23], but the observed distribution may not reflect the true quantitative distribution of these termini in the *CYP19* gene transcripts in a particular cell or tissue.

Our present results employing adipose stromal cells grown in the absence of serum or dexamethasone revealed the presence of equal concentrations of transcripts containing exon I.3 (truncate)- and promoter II-specific sequences. There was no amplification of exon I.4- specific sequences. Results obtained from adipose stromal cells treated with dexamethasone (250 nM) in medium containing 15% FCS are in agreement with our earlier study [23]. Exon I.3 and exon I.4-specific sequences were amplified, but exon I.4-specific sequences were more abundant in dexamethasone-treated cells (Fig. 8). Promoter II-specific sequences were present in very low copy number. Total levels of P450arom transcripts (see coding region, Fig.8, Table 5) were elevated relative to control cells, which is in accordance with our earlier observation that in the presence of serum, glucocorticoids stimulate aromatase activity [32]. Recently, we have shown that the effect of glucocorticoids in the presence of serum is regulated by promoter I.4 [33]. More importantly, we found that the stimulatory action of serum (in the presence of dexamethasone) can be replaced by the cytokine IL-11. Moreover, we have shown that this operates via a Jak/STAT signaling pathway to regulate aromatase P450 gene expression by using promoter I.4 [33]. Therefore, we decided to amplify different transcripts from adipose stromal cells treated with dexamethasone and IL-11. As expected, we observed a high abundance of exon I.4-specific sequences (Table 5). Interestingly, we

also observed amplification of promoter II-specific and exon I.3 (truncate)-specific sequences (Table 5).

In ovarian granulosa cells treated with forskolin, only promoter II-specific sequences were amplified (Fig. 8). Exon I.3 (truncate)-specific sequences were present in very low copy number. The presence of promoter II-specific sequences in cells treated with forskolin is in agreement with our earlier studies based on northern analysis [34]. Presence of exon I.3 (truncate)-specific sequences could not be detected by northern analysis because of low copy number.

It seems likely that promoter II and I.3 are involved in basal transcription of the *CYP19* gene in adipose stromal cells. In cells treated with glucocorticoid and IL-11 or serum, total P450arom transcripts are elevated due to increased expression from exon I.4. However, there are also more exon I.3 (truncate)-specific sequences. So possibly there are other factors which regulate the *CYP19* gene by utilizing promoter I.3. Most interestingly, from these results, we observed that in human adipose stromal cells and ovarian granulosa cells, the exon I.3-specific transcripts being amplified were the truncated version [23]. There was no amplification of full-length exon I.3-specific transcripts. Although the 5′-end primer (RT-2) was chosen from the sequence which was common in both exon I.3 and exon I.3 (truncate) transcripts, the size difference of 106 bp between both amplified PCR products, is very clear. Earlier, by RACE cloning both full-length and truncated exon I.3-specific transcripts were detected in cultured cells [23]. At this point we are unable to explain this difference.

We conclude that by using this very sensitive competitive RT-PCR method, we were able to amplify and quantitate P450arom transcripts present in very low copy number. Most importantly, we were able to differentiate amplified products of only 5-6 bp difference in size. This methodology will be very useful to study the regulation of the *CYP19* gene in different tissues and in different disease states. Experiments are already in progress in our laboratory to determine the levels of the various exon-specific transcripts, present in the endometrium of patients with endometriosis and in adipose tissue of breast cancer patients. Furthermore, this procedure is not only useful for detecting alternatively

spliced transcripts of the *CYP19* gene, but it can in principal be applied to detect multiple transcripts expressed from other genes which are regulated in this fashion.

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FIGURE LEGENDS

Fig. 1 Schematic diagram of the synthesis of internal standard transcripts. Dashed lines depicts the variable region of clones. All numbers in parenthesis represent the oligo number (for sequences see Tables 1 and 2). Black region in oligo# RT-13 and final PCR product, represents the 27 bp random AT-rich insert sequence (loop).

Fig. 2 The effect of the number of PCR cycles on amplification of synthetic transcripts (150 fg). Four reactions were set up, one for each of the synthetic transcripts. Aliquots were collected after the indicated number of cycles and subjected to gel electrophoresis and quantification by means of a Phosphorimager.

Fig. 3 Amplification of synthetic transcripts. Each transcript was amplified using the appropriate specific primers panel (A) as indicated. (B) Each transcript was amplified using coding region-specific primers.

Fig. 4 Cross amplification reactions. Four reactions were set up for each synthetic transcript by using primers specific for coding region, promoter II-specific sequence, exon I.3 and exon I.4, as indicated. Panel A: coding region-specific transcript; Panel B: Promoter II-specific transcripts; Panel C: exon I.3-specific transcripts; Panel D: Exon I.4-specific transcripts.

Fig. 5 Amplification of each transcript from the mixture of three synthetic transcripts (1 pg of each), employing specific primers. Results shown are the mean $\pm$ SD of data from three separate experiments.

Fig. 6 Amplification of mixtures of three transcripts containing varying quantities of promoter II-specific transcripts in the presence of a fixed amount (Panel A: 500 fg; Panel B: 1000 fg) of the other two transcripts i.e. exon I.3 and exon I.4 (see Table 4 for details of concentration of each transcript in the mix).

Fig. 7 Competitive RT-PCR: 1.5 μg of adipose stromal cell RNA was mixed with 0-1 pg of internal standard transcript (coding region-specific) and amplified by using coding region-specific primers. The size of the internal standard amplified product was 221 bp, and that of the target amplified product was 194 bp .

Fig. 8 (I) Amplification of specific 5′-terminal sequences from cDNA of 1 μg of RNA isolated from (A) adipose stromal cells maintained in the absence of dexamethasone and serum; (B) adipose stromal cells in the presence of dexamethasone +serum; (C) ovarian granulosa cells in the presence of forskolin. The specific 5′-terminus being amplified is indicated at the top of each lane. (II) Amplification of GAPDH cDNA from 500 ng RNA isolated from cultured cells. Panels A, B, C are as indicated above.

Table 1. Oligonucleotides used as primers for PCR amplifications

RT-1:	5′ end sense oligo from exon I.4
	5′ GTG ACC AAC TGG AGC CTG 3′
RT-2:	5′ end sense oligo from exon I.3
	5′ GAT AAG GTT CTA TCA GAC C 3′
RT-3:	5′ end sense oligo from Promoter II- specific sequence
	5′ GCA ACA GGA GCT ATA GAT 3′
RT-4:	3′ end antisense oligo from coding exon II
	5′ CAG GCA CGA TGC TGG TGA TG 3′
RT-5:	5′ end sense oligo from untranslated region of exon II
	5′ TCT GAG GTC AAG GAA CAC 3′
RT-6:	3′ end antisense oligo from exon IV
	5′ TTG TTG TTA AAT ATG ATG C 3′
RT-7:	5′ end sense oligo from coding exon II
	5′ TTG GAA ATG CTG AAC CCG AT 3′
RT-8:	3′ end antisense oligo from exon III
	5′ CAG GAA TCT GCC GTG GGG AT 3′
GAPDH1:	5′ end sense oligo from GAPDH gene
	5′ CGG AGT CAA CGG ATT TGG TCG TAT 3′
GAPDH2:	3′ end antisense oligo from GAPDH gene
	5′ AGC CTT CTC CAT GGT GGT GAA GAC 3′

Table 2. Oligonucleotides used as primers for PCR amplification of specific regions of alternate exons I, which were subsequently cloned into the vector

RT-9:	5′ end sense oligo from exon I.4 5′ CUA CUA CUA CUA GTA GAA CGT GAC CAA CTG 3′
RT-10:	5′ end sense oligo from exon I.3 5′ CUA CUA CUA CUA GAT AAG GTT CTA TCA GAC 3′
RT-11:	5′ end sense oligo from Promoter II-specific sequence 5′ CUA CUA CUA CUA GCA ACA GGA GCT ATA GAT 3′
RT-12:	5′ end sense oligo from coding exon II 5′ CUA CUA CUA CUA TTG GAA ATG CTG AAC CCG AT 3′
RT-13:	3′ end antisense oligo from exon III containing a 27 bp random AT rich insert 5′ CAU CAU CAU CAU CAC AGG AAT CTG CCG TGG GGA TGA GGG GTC CAA TTC AAA TAT TTA TAT TAA AAT TAT TTT AAA CCA TGC AGT AGC CAG 3′

Note: Underlined nucleotides represent a 27 bp random AT rich sequence inserted in the oligonucleotide.

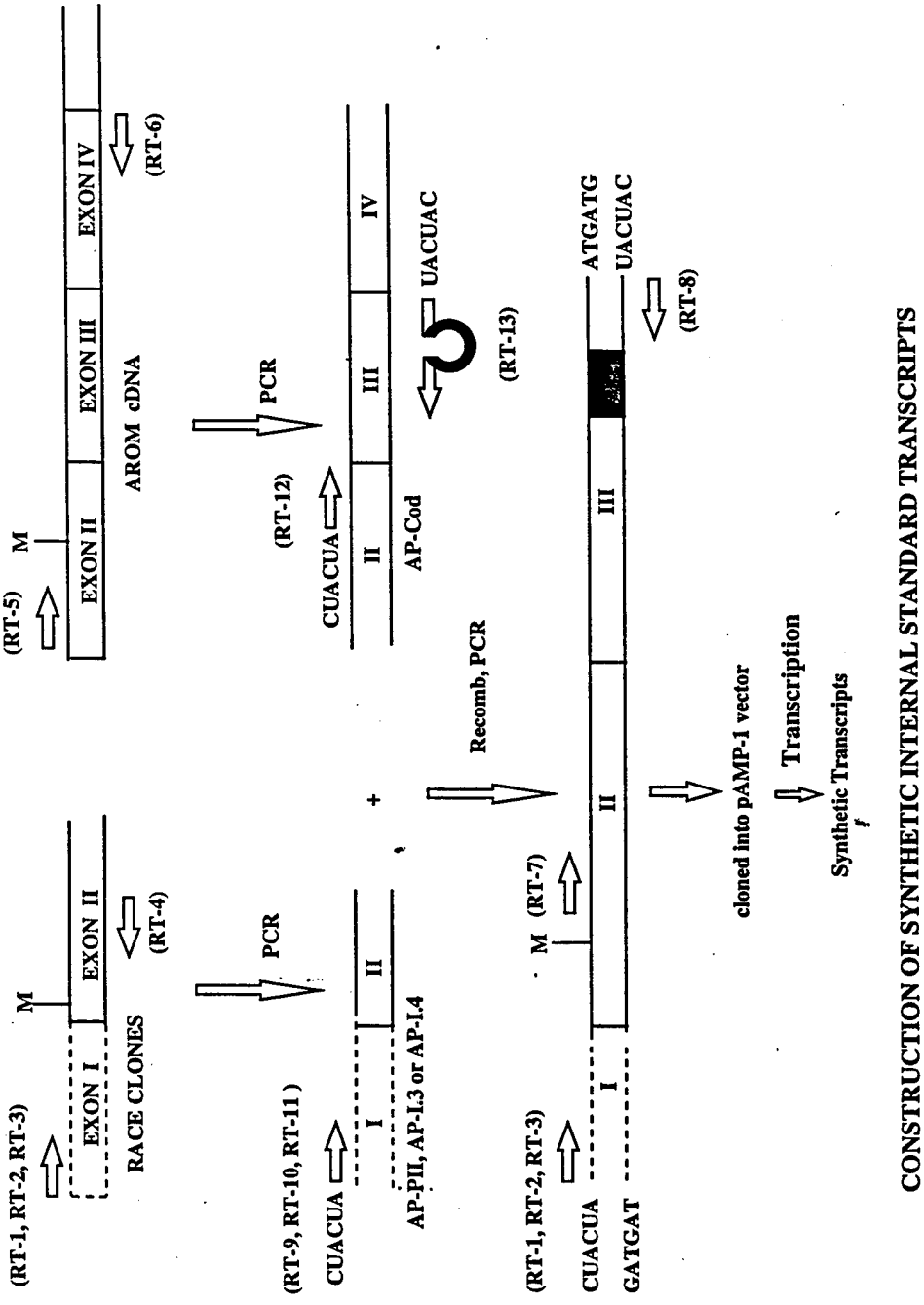
Table 3. Expected sizes of PCR amplified products

Alternate Exon I	Target	Internal Standard
Exon I.4 -- Exon III	294 bp	321 bp
Exon I.3 -- Exon III	395 bp	422 bp
Exon I.3 (truncate)-- Exon III	289 bp	
Exon II -- Exon III (PromoterII-specific)	305 bp	332 bp
Exon II -- Exon III (coding region)	194 bp	221 bp
GAPDH	306 bp	

Table 4. Conditions used to standardize quantification of Internal standard

Standard experiments	Amounts of Exon specific synthetic transcripts			
	Promoter II-specific	Exon I.3	Exon I.4	Sheep Kidney
1	50-1000 fg	50 fg	50 fg	50 ng
2	50-1000 fg	500 fg	500 fg	50 ng
3	50-1000 fg	1000 fg	1000 fg	50 ng
4	50 fg	50-1000 fg	50 fg	50 ng
5	500 fg	50-1000 fg	500 fg	50 ng
6	1000 fg	50-1000 fg	1000 fg	50 ng
7	50 fg	50 fg	50-1000 fg	50 ng
8	500 fg	500 fg	50-1000 fg	50 ng
9	1000 fg	1000 fg	50-1000 fg	50 ng

Figure 1



CONSTRUCTION OF SYNTHETIC INTERNAL STANDARD TRANSCRIPTS

Figure 2

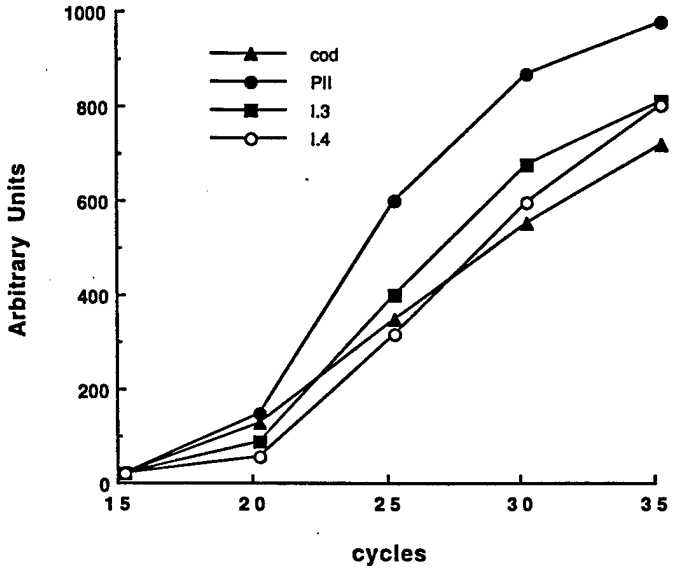

1000
800
600
400
200
0
Arbitrary Units
cod
PII
I.3
I.4
15
20
25
30
35
cycles

Figure 3

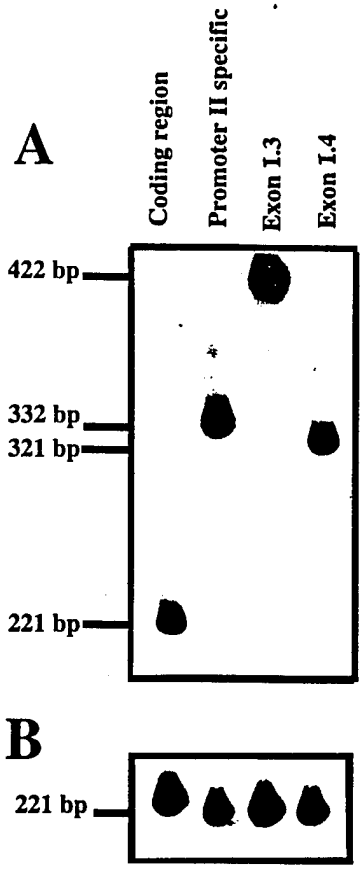

A
Coding region
Promoter II specific
Exon I.3
Exon I.4
422 bp
332 bp
321 bp
221 bp
B
221 bp

Figure 4

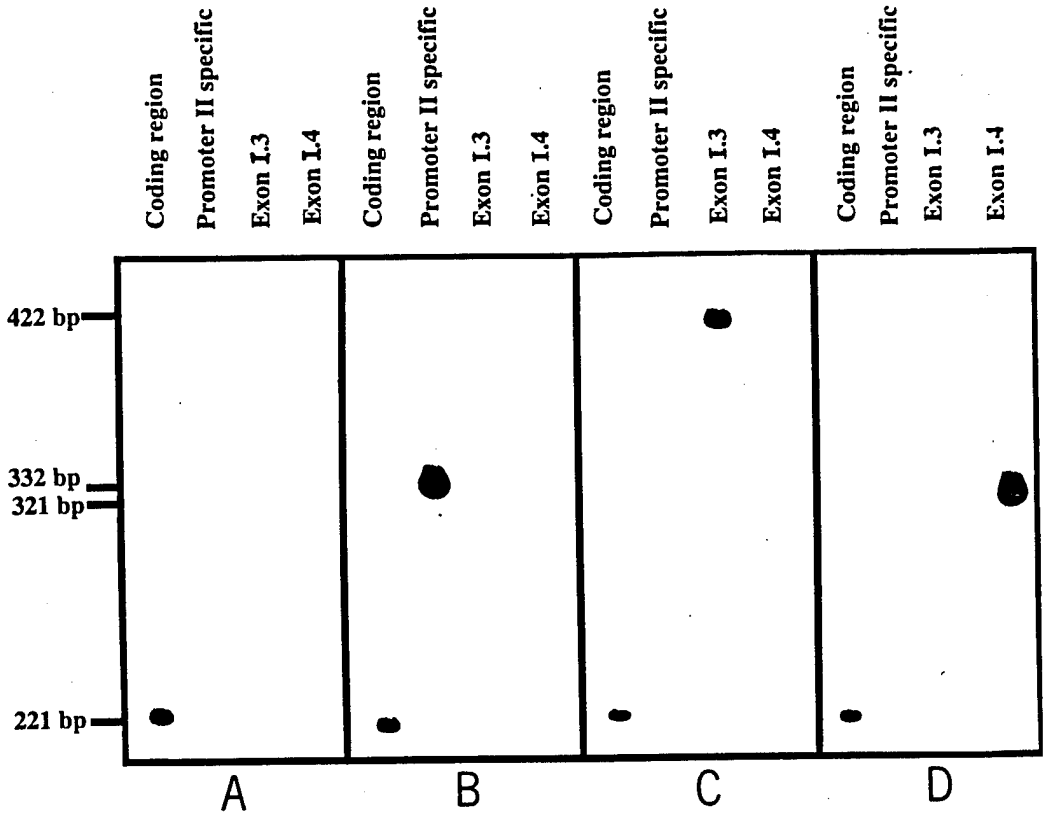

Coding region
Promoter II specific
Exon I.3
Exon I.4
Coding region
Promoter II specific
Exon I.3
Exon I.4
Coding region
Promoter II specific
Exon I.3
Exon I.4
Coding region
Promoter II specific
Exon I.3
Exon I.4
422 bp
332 bp
321 bp
221 bp
A
B
C
D

Figure 5

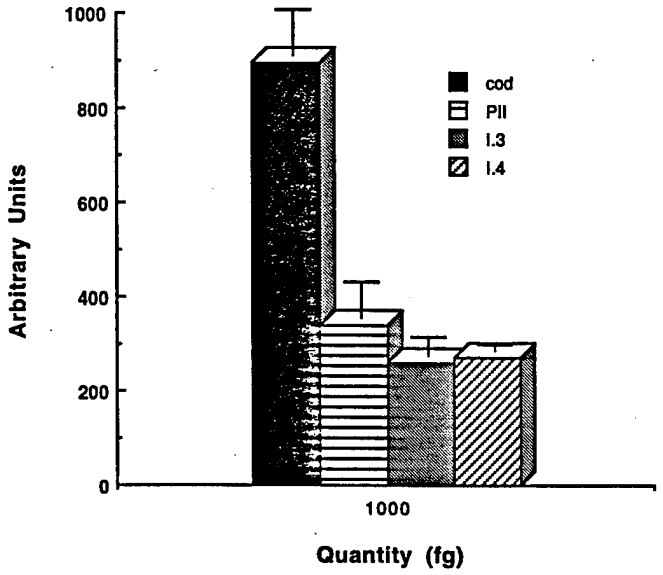

1000
800
600
400
200
0
Arbitrary Units
cod
PII
I.3
I.4
1000
Quantity (fg)

Figure 6 a

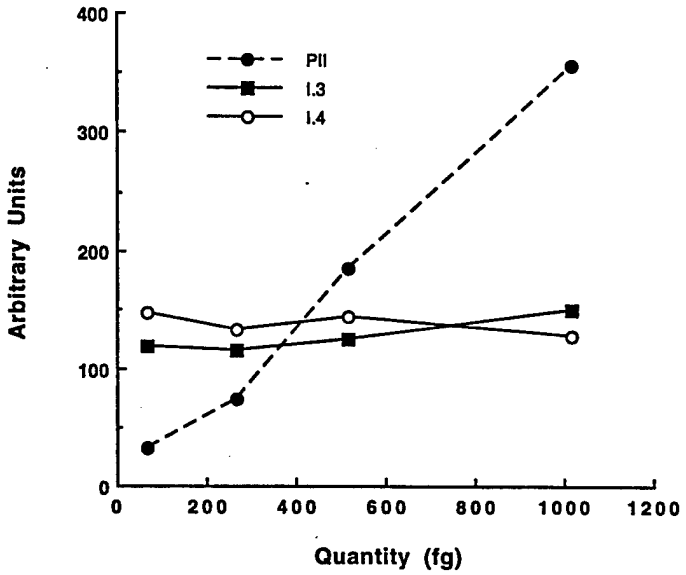

400
300
200
100
0
Arbitrary Units
PII
I.3
I.4
0
200
400
600
800
1000
1200
Quantity (fg)

Figure 6b

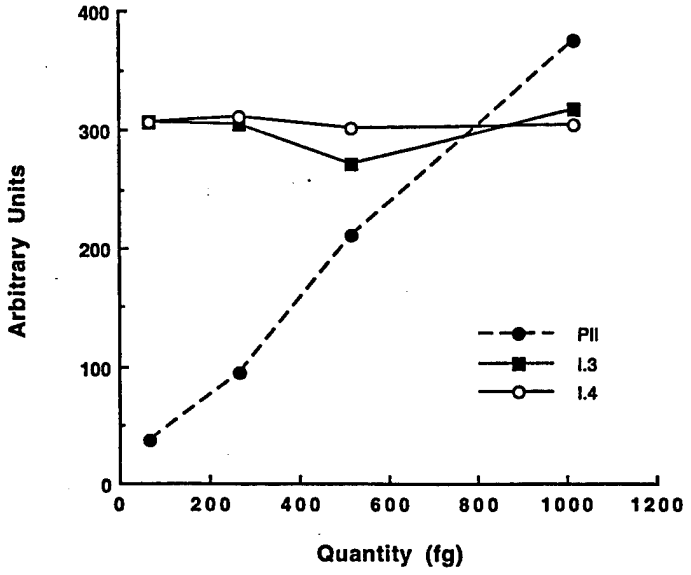
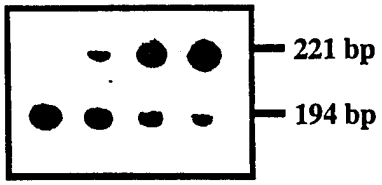

400
300
200
100
0
Arbitrary Units
0
200
400
600
800
1000
1200
Quantity (fg)
PII
I.3
I.4

Figure 7

1.5 1.5 1.5 1.5 Target RNA (ug)

- 0.1 0.5 1.0 Internal Standard (pg)



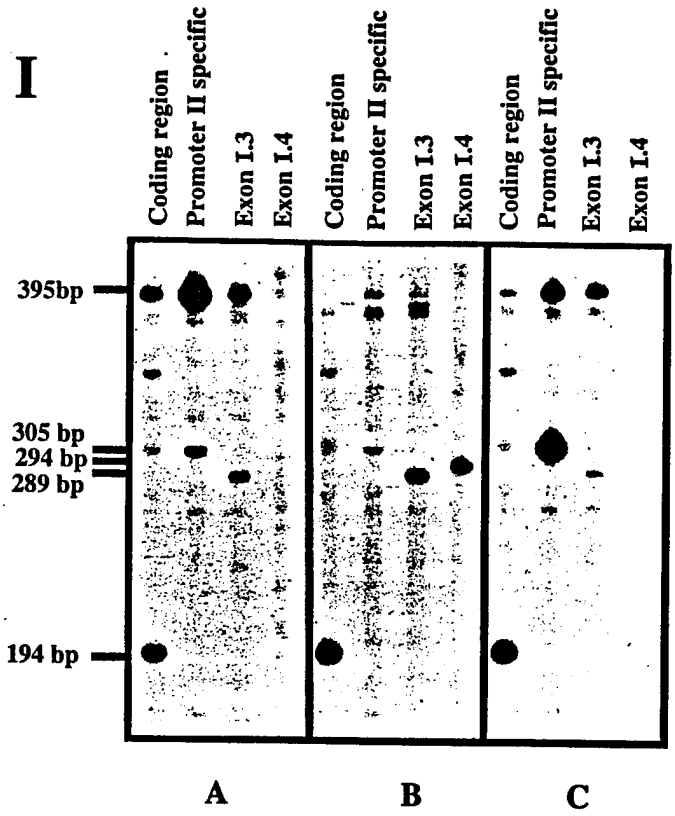


Figure 8 I

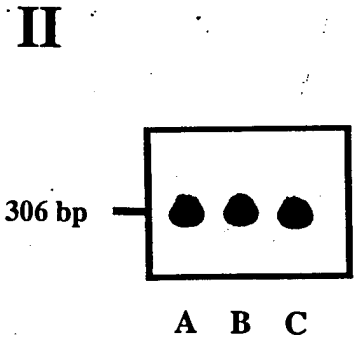


Figure 8 II